Chapter 24

Tissue Extracts for Quantitative Mass Spectrometry of Planarian Proteins Using SILAC

Alexander Böser, Hannes C. A. Drexler, and Kerstin Bartscherer

Abstract

SILAC (stable isotope labeling by amino acids in cell culture) proteomics enables the relative quantification of proteins in one or more biological samples by mass spectrometry. This technology is based on the metabolic incorporation of heavy isotope-labeled essential amino acids into nascent proteins in vivo. Here, we describe the preparation of SILAC protein samples from planarians, flatworms with high regenerative potential and tissue plasticity. Applications for SILAC proteomics of planarians include the analysis of protein abundances, protein–protein interactions and turnover rates during stem cell-based regeneration and tissue homeostasis.

Key words Planarians, SILAC, Quantitative proteomics, Protein extraction, Protein processing, FASP

1 Introduction

Changes in gene expression during different stages of planarian regeneration or after gene-specific knockdown have been mainly analyzed by microarray methods or, more recently, by next-generation sequencing. This has resulted in the identification of hundreds of genes, some with important functions in planarian regeneration and homeostasis [1–5]. However, transcriptome analyses do not account for posttranscriptional regulatory mechanisms that may influence protein levels. Quantitative proteomics, in contrast, can be used to directly measure and compare protein levels in diverse biological samples. Among the most sensitive and accurate quantitative methods for protein analysis is stable isotope labeling by amino acids in cell culture (SILAC) proteomics, which involves the incorporation of heavy isotope amino acids into nascent proteins and their subsequent analysis on the peptide level by mass spectrometry. In this approach, cells are fed with media in which an essential amino acid, such as $^{12}C_6$ lysine, is replaced by its heavy isotope labeled equivalent ($^{13}C_6$ lysine). After a few cell divisions, all of a cell's proteins contain this heavy isotope, constituting a fully labeled "heavy" proteome [6].

Jochen C. Rink (ed.), *Planarian Regeneration: Methods and Protocols*, Methods in Molecular Biology, vol. 1774,
https://doi.org/10.1007/978-1-4939-7802-1_24,

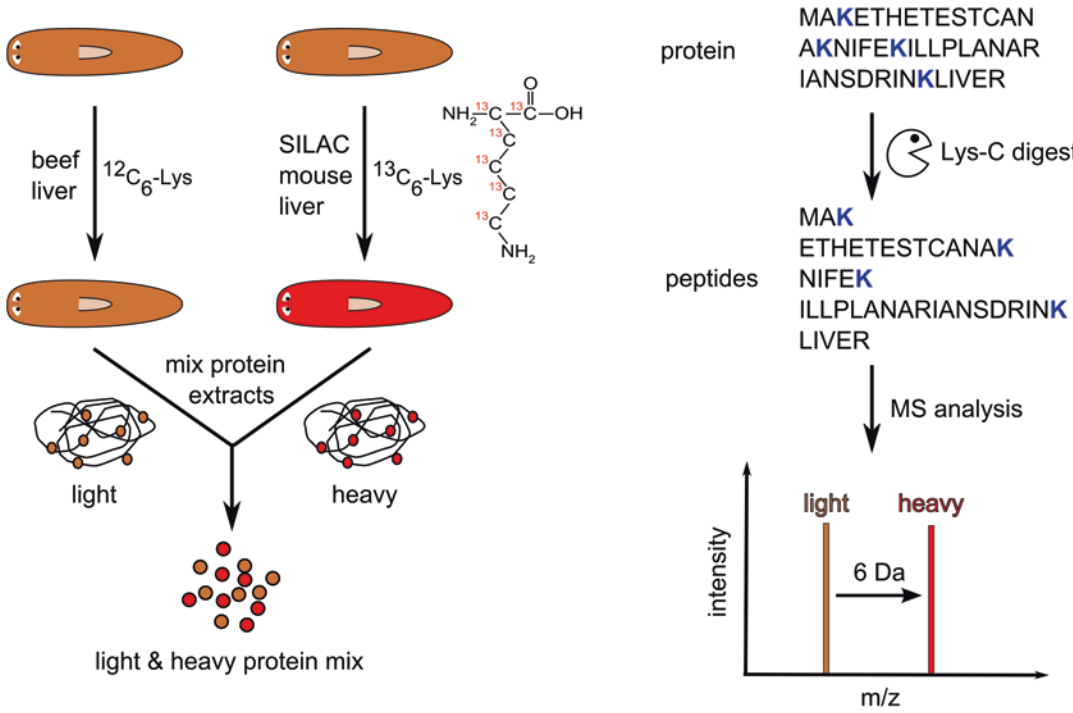

Fig. 1 Planarian labeling using SILAC. Planarians are fed with $^{13}C_6$ labeled SILAC mouse liver. Protein extracts from unlabeled (light) and labeled (heavy) planarians are mixed 1:1, resulting in a protein mix containing equal amounts of light and heavy proteins. Proteins are then digested with the endoproteinase Lys-C, cleaving proteins at the C-terminus after each lysine residue. Mass spectrometry (MS) analysis enables comparisons between the light and heavy isotope cluster intensities of each peptide and the precise determination of their relative quantity in each of the experimental samples

For relative quantification of protein levels in two biological samples, two cell populations are either grown in the "heavy" medium (supplemented with $^{13}C_6$ lysine) or in normal ("light") medium. When the desired labeling efficiency has been reached, the proteins of both populations are extracted and mixed in a defined ratio, and cleaved by lysyl endoproteinase (Lys-C) at the C-terminus of each lysine residue. This ensures that all but the C-terminal peptides derived from the "heavy" cell population contain at least one labeled lysine (+6 Da). Peptides are subsequently analyzed by mass spectrometry. As a "heavy" peptide behaves like its natural counterpart during sample preparation and chromatography, yet its isotope composition and hence its mass-to-charge ratio is different, the two isotope peaks elute together but are easily distinguishable in the mass spectrum (Fig. 1). Using appropriate software, the relative abundance of the two peptides can then be calculated, enabling precise relative quantification.

While SILAC proteomics was first established for cultured cells, it has been also applied to whole organisms such as mice [7], *Drosophila* [8], zebrafish [9], salamanders [10] and nematodes

[11]. Recently, we reported on the generation of SILAC planarians [12]. To achieve full labeling of *Schmidtea mediterranea*, a planarian species with great potential to regenerate all missing body parts, animals were fed once per week with a slice of SILAC mouse liver [7], resulting in a labeling efficiency of 97% after 20 weeks.

By analyzing the proteomes of labeled and normal planarian cells and tissues with mass spectrometry, a quantitative description of relative protein levels can be achieved. With the help of a SILAC spike-in standard [13], a "heavy" protein extract from the species/tissue under investigation, several "light" biological samples can be independently analyzed, enabling the comparison of relative protein levels in multiple samples. This is particularly useful as labeling is carried out only once and is not required for each experimental condition separately. Hence, a SILAC spike-in standard may be applied to study changes in protein expression during different stages of regeneration or after various RNAi or drug treatments in multiple experiments.

As the rate of amino acid incorporation into proteins depends on the rate of protein synthesis, SILAC proteomics can also be used to investigate protein turnover. While a fast protein turnover results in proteins with a high degree of labeling after a short labeling period, the labeling of slow turnover proteins requires more time. Other applications for SILAC planarians may include the quantitation of post-translational modifications or the analysis of

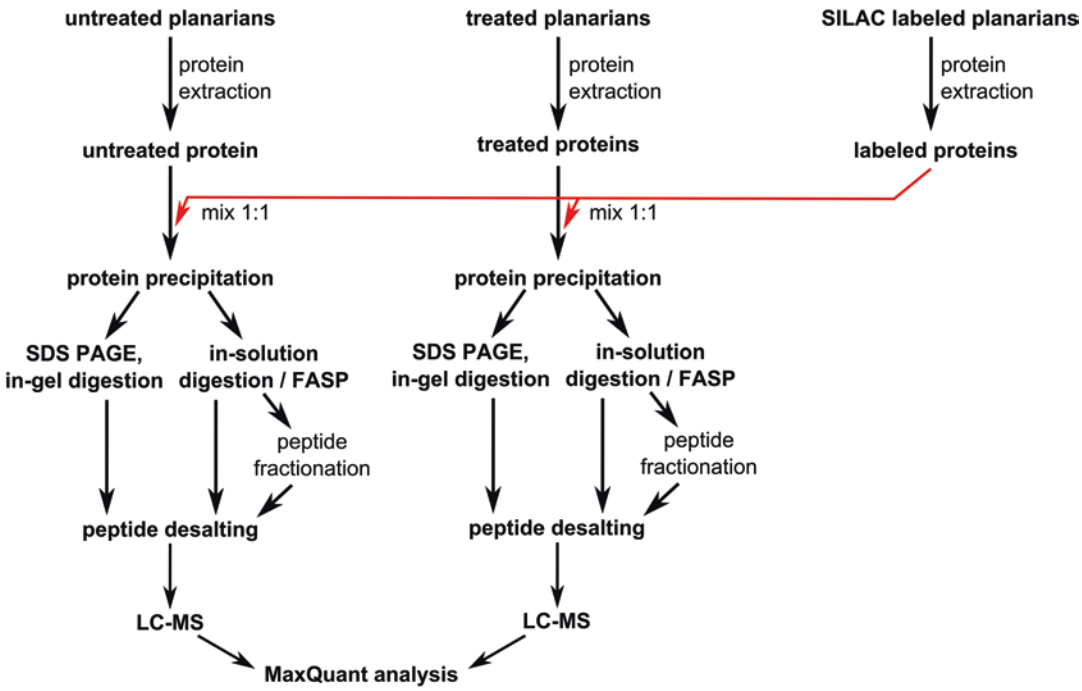

Fig. 2 Workflow for SILAC spike-in proteomics in planarians. *FASP* filter-aided sample preparation, *LC-MS* liquid chromatography-coupled mass spectrometry analysis

protein complexes, similar to approaches taken for other model systems [14, 15]. SILAC proteomics of planarians, therefore, will enable the analysis of protein abundance and turnover as well as protein–protein interactions and may greatly contribute to our understanding of planarian regeneration and tissue turnover.

Here we provide a detailed protocol for the generation of protein samples from SILAC planarians that may be used directly for mass spectrometry (*see* workflow in Fig. 2). We briefly describe the labeling of planarians and then focus on two methods for protein extraction and processing (filter-aided sample preparation (FASP) and in-gel preparation). Although this protocol has been optimized for the planarian species *Schmidtea mediterranea*, it may be similarly applicable to other planarian species.

2 Materials

2.1 Planarian Maintenance and Labeling

1. Planarian artificial water (PAM): 1.6 mM NaCl, 1 mM $CaCl_2$•$2H_2O$, 1 mM $MgSO_4$, 0.1 mM $MgCl_2$, 0.1 mM KCl, 1.2 mM $NaHCO_3$ for planarian maintenance.
2. $^{13}C_6$ lysine labeled SILAC mouse liver (e.g., from Silantes, Germany). Cut frozen SILAC mouse liver into slices (1 mm thickness and 3–5 mm length) in a petri dish on ice and transfer individual slices into 0.5 ml PCR tubes. Store at −80 °C for up to several months.
3. "Normal" mouse liver. Process as above.

2.2 Protein Extraction

1. Planarian lysis buffer (modified RIPA buffer): 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1% DTT prepared in 50 mM Tris–HCl pH 7.5 (stored at 4 °C). One protease inhibitor cocktail tablet (EDTA free, Roche) is added per 10 ml directly before use.
2. Polypropylene pestles for tissue homogenization. A cordless motor (e.g., Sigma) is useful when processing many samples.
3. Benzonase for DNA/RNA degradation.

2.3 Protein Precipitation and In-Solution Digestion

1. 100% acetone, ice cold.
2. Resuspension buffer: 6 M urea, 2 M thiourea, 20 mM Hepes pH 7.5; store aliquots at −20 °C.
3. Ammonium bicarbonate (ABC) solution: 50 mM ABC in H_2O; store aliquots at −20 °C.

Endoproteinase Lys-C (e.g., Wako Chemicals; prepare stock solution by dissolving the content of the vial in ABC to a final concentration of 0.5 µg/µl and store in 10 µl aliquots at −80 °C). Use about 0.5 µg per gel slice (SDS-PAGE) or 1 µg per 100 µg of protein (in-solution digest).

2.4 In-Solution Digestion

1. Dithiothreitol (DTT) stock solution: 1 M DTT in MilliQ (Millipore) H_2O. Store aliquots frozen at −20 °C.
2. Iodoacetamide (IAA) stock solution: 550 mM IAA in MilliQ H_2O. Prepare and freeze in small aliquots (10–50 μl) and store at −20 °C to avoid multiple freeze–thaw cycles.
3. Solution C: 5% acetonitrile (ACN), 1% trifluoroacetic acid (TFA). Prepare by mixing HPLC grade reagents with water in the appropriate proportions.

   Safety note: TFA is a very strong acid. Wear safety glasses and prepare the solution in a fume hood. First add the appropriate amount of MQ H_2O to the container, then the acid. Use glassware to pipet TFA.

2.5 FASP/Post-FASP

1. Amicon ultra 30K filter units for filter-aided sample preparation (FASP) (e.g., Millipore).
2. Iodoacetamide (IAA) stock solution: 550 mM IAA in MilliQ H_2O. Prepare small aliquots (10–50 μl) and store at −20 °C to avoid multiple freeze–thaw cycles.
3. NaCl solution: 0.5 mM NaCl in MQ H_2O. Store aliquots frozen at −20 °C.
4. Acidifying Solution: 2% TFA in MQ H_2O. Safety note TFA: *see* above.
5. HP extraction disc cartridges (e.g., Empore 3M) for desalting of FASP generated peptides.
6. Additional reagents: methanol (HPLC grade), ACN (HPLC or LC-MS grade), Britton & Robinson universal buffers pH 11, 8, 6, 5, 4, 3, 2.
7. Additional solutions: 0.1% TFA (*see* Safety note TFA), 70% ACN, 0.1% TFA/70% ACN.
8. Anion exchange-SR extraction disks (e.g., Empore 3M) for fractionation by anion exchange chromatography, if desired.

2.6 SDS-PAGE and In-Gel Digestion

1. Coomassie (Bradford) protein assay for estimation of protein concentrations.
2. Bis-Tris gels for SDS–polyacrylamide gel electrophoresis (SDS-PAGE) with 2-morpholino ethane sulfonic acid (MES) buffer, sample buffer and reducing agent. Our laboratory uses the commercially available NuPAGE® Novex 4–12% gel system (Invitrogen).
3. SDS-PAGE gel fixation solution: 40% methanol, 10% acetic acid.
4. Novex Colloidal Blue staining kit (Pierce/Thermo Scientific) for SDS-PAGE gel staining.
5. Washing solution: 50 mM ABC–50% ethanol.

6. DTT working solution (DTTw): 10 mM DTT/50 mM ABC. Prepare freshly by diluting the DTT stock solution with 50 mM ABC.
7. IAA working solution (IAAw): 55 mM IAA/50 mM ABC. Prepare freshly by diluting the IAA stock solution with 50 mM ABC.
8. Additional reagents: ethanol (absolute), ACN (HPLC/LC-MS grade).
9. Additional solutions: 30% ACN/3% TFA, 70% ACN, Solution C (5% ACN/1% TFA).

2.7 Stage Tipping

1. Solid phase extraction disk C18 material (3M Empore).
2. Blunt luer lock needle (e.g., Hamilton # 90516, 16 gauge or similar) and the plunger of a 50 µl glass syringe (e.g., Hamilton, Plunger Assembly for 1705 syringe; # 1162-01 or similar).
3. Methanol (HPLC grade).
4. Solution A: 0.5% acetic acid in MQ H_2O.

   Safety note: Acetic acid is a strong acid. Wear safety classes and prepare the solution in a fume hood. First add the appropriate amount of MQ H_2O to the container, then the acid. Use glassware to pipet acetic acid.
5. Solution B: 80% ACN–0.5% acetic acid in MQ H_2O.
6. Solution C: 5% ACN–1% TFA.

2.8 Mass Spectrometric Data Analysis

The MaxQuant software package (version 1.3.0.5 or higher) and its built-in Andromeda search engine are suitable for identification and quantification of peptides, Perseus for statistical analysis [16, 17]. In silico translated transcriptome datasets, such as the SRG12 proteome [12], can be implemented in the pipeline and used for protein identification.

3 Methods

3.1 Planarian Maintenance and Labeling

1. Maintain asexual planarians of the species *Schmidtea mediterranea* (BCN-10) in PAM in plastic containers (Tupperware) at 20 °C in the dark. Feed once a week with either normal mouse ("light") or SILAC mouse ("heavy") liver.
2. For feeding, thaw a sufficient number of mouse liver aliquots and add them to the culture container for 1–2 h (*see* **Note 1**). We typically use three slices to feed 20 planarians (8–12 mm in size).
3. Wash planarians after feeding by removing remaining tissue pieces with a plastic Pasteur pipette and rinsing with cold tap water. Add fresh PAM after washing.

4. For generating SILAC standards, feed planarians at least once per week for at least 20 weeks to ensure high labeling efficiency (*see* **Note 2**).

3.2 Protein Extraction

All extraction steps must be carried out on ice (*see* **Note 3**).

1. Collect planarians and transfer them into a 1.5 ml low protein binding reaction tube. Carefully remove all remaining PAM.
2. Dip the tube into liquid nitrogen for 5–10 s to snap-freeze the animals.
3. Add ice-cold Planarian lysis buffer (approximately 150 µl for five planarians at a size of 8 mm) and mechanically homogenize the tissue using a pestle.
4. Add 1.5 µl Benzonase/sample for the digestion of DNA and RNA and incubate in a thermo-shaker at 1400 rpm and 4 °C for 1 h.
5. Spin samples at 15,000 rcf, 4 °C for 15 min to pellet insoluble cell debris.
6. Collect the supernatant containing the proteins to be analyzed and transfer to a new low protein binding tube.
7. Determine the protein concentration by preparing a 1:10 and 1:20 dilution of the supernatant in water and using the Coomassie (Bradford) assay (*see* **Note 4**).
8. Store samples at −80 °C until use. Protein extracts from SILAC planarians may be frozen as small aliquots to be used as a spike-in standard in subsequent experiments.

3.3 Protein Precipitation

1. Mix heavy and light protein samples at a ratio of 1:1 (amount of protein).
2. Transfer the desired amount of protein mixture (50–200 µg total protein) into a new 1.5 ml low protein binding reaction tube and add four times the volume of ice-cold acetone (*see* **Note 5**).
3. Vortex briefly and then incubate for at least 60 min (preferably overnight) at −20 °C.
4. Spin at 15,000 rcf and 4 °C for 10 min to pellet the precipitated proteins.
5. Remove supernatant and briefly air-dry pellet at RT for about 3 min (*see* **Note 6**).
6. Resuspend protein pellet with the desired volume of Resuspension buffer (about 10 µl for SDS-PAGE, 50 µl for in-solution digestion, 100 µl for FASP) and directly continue with protein processing steps below. *See* **Note 7** for an alternative precipitation method.

3.3.1 Protein Processing for MS Analysis

Subsequent to this step, protein samples may be processed for MS analysis by one of three methods: In-solution digestion (Subheading 3.4), filter-aided sample preparation (FASP, Subheading 3.5) or SDS-PAGE followed by in-gel digestion (Subheading 3.6). The in-solution digestion method is fast and especially suitable for the a priori analysis of labeling efficiency or the validation of mixing ratios of combined light and heavy proteins (*see* **Note 8**). However, the method typically allows identification and quantification of comparably few proteins due to the complexity of the peptide mixtures. Experimental samples are therefore typically analyzed by either the FASP or in-gel digestion protocols, which both include fractionation steps to reduce sample complexity and thus yield higher protein identification and quantification efficiencies. Protein immobilization on a filter unit in the FASP procedure allows the processing of high protein content samples (milligram range) while simultaneously removing undesired buffer components (SDS, Triton X-100) and enabling the analysis of proteins that are difficult to separate by SDS-PAGE [18]. Peptides resulting from FASP can be further fractionated by ion exchange chromatography as described below. Similarly, SDS-PAGE can be used to remove unwanted buffer components, yet the capacity of the gel can be limiting in some applications (100 µg of protein/lane should not be exceeded). Size fractionation can be achieved by cutting gel lanes into several slices, whereby more slices increase sensitivity at the cost of longer MS measurement times. If the amount of sample is not limiting (>150 µg of protein) we recommend to split the sample and analyze it with both methods (e.g., 100 µg of protein for FASP, and 50 µg for SDS-PAGE with in-gel digestion).

3.4 In-Solution Digestion

Carry out all procedures at room temperature unless stated otherwise.

1. Add DTT stock solution to a resuspended protein sample (Subheading 3.3, **step 6**) to a final concentration of 10 mM. Incubate at 37 °C for 1 h to reduce disulfide bonds.
2. Add IAA stock solution to a final concentration of 55 mM. Incubate at room temperature for 30 min in the dark to alkylate previously reduced Cys residues and to prevent the reformation of disulfide bonds.
3. Add 1 µg of Lys-C. Digest protein samples at 37 °C overnight.
4. Dilute the sample 1:1 with Solution C. Remove urea from the resuspended sample by stage tipping (*see* Subheading 3.7).

3.5 FASP

Carry out all procedures at room temperature unless stated otherwise.

1. Transfer a resuspended protein sample (Subheading 3.3, **step 6**) to the filter unit (*see* **Note 9**).

2. Spin at 14,000 rcf until all solution passed through the filter. Discard the flow-through.
3. Add 100 µl Resuspension buffer to the filter unit.
4. Add 10 µl of 550 mM IAA Solution to the filter unit and incubate for 20 min in the dark. This step alkylates the proteins.
5. Add 100 µl Resuspension buffer to the filter unit.
6. Spin at 14,000 rcf for 10 min. Discard flow-through.
7. Repeat **steps 4** and **5** twice more.
8. Add 100 µl 50 mM ABC to the filter unit.
9. Spin at 14,000 rcf for 10 min. Discard flow-through.
10. Repeat **steps 8** and **9** twice more.
11. Transfer the filter unit to a new low protein binding reaction tube.
12. Add Lys-C (1 µg per 100 µg of protein in 40 µl 50 mM ABC solution) to the filter unit.
13. Briefly shake the tube at 600 rpm on a thermo-shaker.
14. Incubate the filter unit at 37 °C in a cell culture incubator (100% humidity) overnight to allow digestion of the immobilized proteins.
15. The next day, remove the filter unit from the incubator and add 50 µl of NaCl solution.
16. Centrifuge at 14,000 rcf for 10 min. During centrifugation digested peptides pass through the filter and are collected in the tube.
17. Dispose of the filter unit and acidify the peptide solution (flow-through) by adding 15 µl of Acidifying Solution per 100 µl of sample (*see* **Note 10**). Store at RT while conditioning the extraction disk cartridge for desalting.
18. Cartridge conditioning: Add 1 ml methanol and spin at 1500 rcf for 1 min. Add 500 µl 0.1% TFA/70% ACN and spin at 1500 rcf for 1 min. Add 500 µl of 0.1% TFA and spin at 1500 rcf for 1 min.
19. Load the sample on the freshly conditioned cartridge and spin at 150–300 rcf for 3 min (time may vary depending on the sample amount; spin until all sample has passed through).
20. Wash the cartridge with 500 µl 0.1% TFA and spin at 300 rcf for 3 min (until all solution has passed through).
21. Transfer cartridge into a new 15 ml Falcon tube and elute with 500 µl 70% ACN by spinning at 300 rcf for 3 min.
22. Transfer solution into a low-bind reaction tube.
23. Evaporate the supernatant in a speedvac (vacuum concentrator) and store the desalted peptides at −20 °C, or directly resuspend for fractionation (*see* **Note 11**) prior to LC-MS analysis.

3.6 SDS-PAGE and In-Gel Digestion

Carry out procedures at room temperature unless stated otherwise.

1. Add a suitable SDS-Page sample buffer + reducing agent to the resuspended protein sample (Subheading 3.3, **step 6**). Using the commercial NuPAGE® system, we usually resuspend the protein pellet in 12 µl of Resuspension buffer (Subheading 3.3, **step 6**), then add 4 volumes of sample buffer and 1.6 µl of reducing agent.
2. Incubate the sample at 70 °C for 10 min to reduce disulfide bonds.
3. Separate the proteins on an SDS-PAGE gel (4–12% Bis-Tris gel) at 200 V for approximately 30 min.
4. Remove the gel from the electrophoresis apparatus and transfer into SDS-PAGE gel fixation solution. Fix for 10 min under gentle agitation.
5. Transfer the fixed gel into Novex Colloidal Blue staining solution A for 10 min, followed by the addition of solution B and an incubation of 3–12 h (overnight; *see* **Note 12**).
6. Wash the gels with at least three exchanges of water during an interval of about 2 h.
7. Place the gel on a glass or plastic plate. Use a clean scalpel to first separate individual lanes, then cut each lane into 11–15 fragments (*see* **Note 13**).
8. Dice each fragment into small pieces to maximize digestion efficiency (cubes of approx. 1 mm^3 volume) and transfer pieces into a separate 1.5 ml low protein binding reaction tube. Label the tubes according to the experiment/lane and slice number.
9. Add 100 µl Washing solution to the gel pieces and incubate for 20 min.
10. Carefully remove solution by aspiration with a P200 pipette.
11. Repeat **steps 9** and **10** one more time.
12. Add 100 µl of 100% ethanol and incubate for 10 min to dehydrate the gel pieces.
13. Remove the supernatant and dry gel pieces using a speedvac.
14. Add 100 µl DTTw solution and incubate on a thermo-shaker at 900 rpm, 56 °C for 45 min. This step reduces the protein content of the gel pieces.
15. Remove the supernatant, add 100 µl of IAAw solution and incubate for 30 min in the dark. This step alkylates the protein content of the gel pieces.
16. Remove the supernatant and wash with 100 µl 50 mM ABC for 10 min.
17. Remove the supernatant and dehydrate the gel pieces by adding 100 µl 100% ethanol for 10 min.

18. Repeat **steps 16** and **17** one more time.
19. Prior to digestion, dehydrate gel pieces by adding 100 µl 100% ethanol for 10 min.
20. Remove the supernatant and completely dry gel pieces in a speedvac. Gel pieces appear as whitish particles at this stage.
21. Add 0.5 µg of Lys-C in 40 µl 50 mM ABC solution to the sample. After 15 min, check if all rehydrated gel pieces are still covered by liquid. If gel pieces are not covered, add an additional volume of up to 40 µl 50 mM ABC solution (without Lys-C). Incubate tubes at 37 °C overnight. This step digests the protein content of the gel pieces and already liberates some of the resulting peptides into the supernatant.
22. The next day, carefully collect the supernatant (elution 1) and transfer into a new 1.5 ml low protein binding tube.
23. Add 100 µl of 30% ACN/3% TFA to the residual gel pieces and incubate for 20–30 min. Collect the supernatant (elution 2) and pool with previous elution. When processing multiple samples in parallel, take care to only pool eluates originating from the same gel fragment.
24. Add 100 µl 70% ACN and incubate for 20–30 min. Collect the supernatant pool with previous elutions.
25. Repeat **step 25**.
26. Add 100 µl 100% ACN and incubate for 20–30 min. Collect the supernatant and pool with previous elutions.
27. Repeat **step 27**.
28. Evaporate the collected supernatants in a speedvac.
29. Dissolve the lyophilized peptides in 20 µl Solution C.
30. Load on stage tips (Subheading 3.7) for desalting and storage.

3.7 Stage Tipping

Stage tipping is an efficient method to desalt peptides prior to MS analysis [19]. Furthermore, peptides can be safely stored immobilized on the tips at 4 °C until the MS analysis can be performed. Stage tipping involves the construction of a simple microcolumn with hydrophobic C18 filter material, which retains the peptides solubilized in Solution C (Subheading 3.6, **step 30**) upon passage through the filter, while salts and other hydrohilic contaminants pass through. Bound peptides are then recovered by elution with a high concentration of organic modifier (80% ACN) in the elution buffer.

1. Use the blunt luer lock needle to punch out round disks from the C18 material.
2. Transfer a total of three disks into a P200 pipette tip by pushing the filter discs out of the needle and into the tip by using an appropriate plunger and compress them slightly (*see* **Note 14** and Fig. 3a).

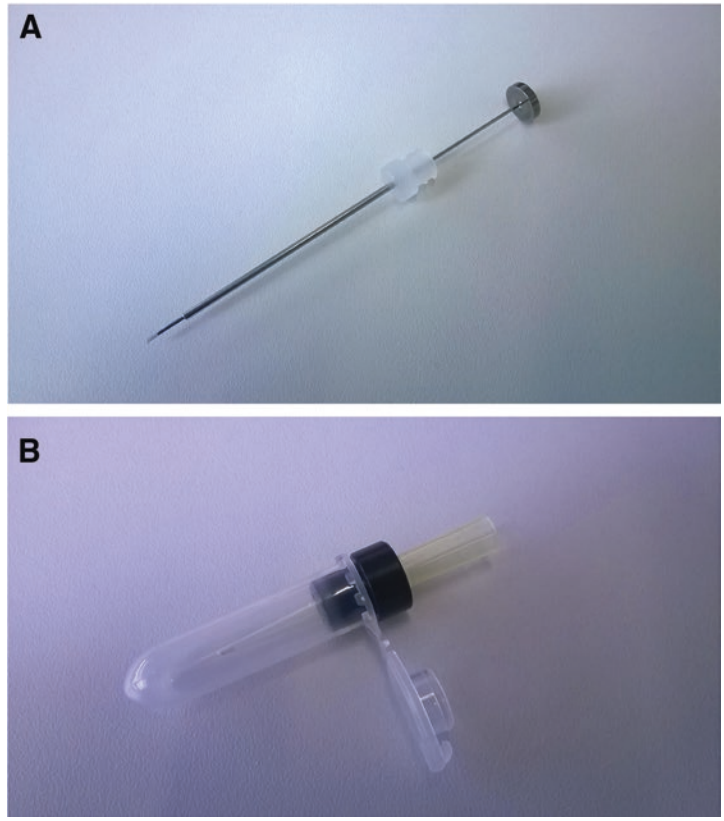

Fig. 3 Custom-made stage tipping tools. (**a**) Stage tip assembly. (**b**) Centrifugal unit

3. Insert the filter tip via appropriate adapters (custom-made) into a 2 ml Eppendorf cup (Fig. 3b).
4. Precondition the C18 filter discs by adding 20 µl of methanol and spin at 800 rcf for 2 min.
5. Add 20 µl of Solution B and spin at 800 rcf for 2 min.
6. Wash filters by adding 20 µl of Solution A and spin at 800 rcf for 2 min.
7. Repeat **step 6**.
8. Add sample to stage tip and spin at 800 rcf until all of the liquid has passed through.
9. Add 20 µl of Solution A and spin at 800 until all solution has passed through.
10. Discard flow-through.
11. Store stage tips at this step in a refrigerator at 4 °C until elution and MS analysis.
12. Add 20 µl of Solution B and use air pressure (syringe) to pass the solution through the stage tip. Collect solution containing

the peptides in appropriate container (96 well place, glass/plastic vials compatible to the sample collector of your liquid chromatography system).

13. Repeat **step 10**.
14. Reduce sample volume to 2–3 μl in speedvac (takes about 45 min).
15. Add 8 μl of Solution A. The sample can directly be used for LC-MS analysis.

4 Notes

1. Planarians having fed on mouse liver can be easily distinguished by the reddish color of their intestine. If required, this criterion can be used to sort out animals that have not eaten.
2. To monitor labeling efficiency over time, we recommend weekly protein extractions from 2–3 planarians during the feeding period. Perform an in-solution digestion with approximately 7 μg of protein and determine the labeling efficiency with the following formula after mass spectrometry: (Ratio H/L × 100)/(Ratio H/L + 1). The Ratio H/L is directly provided in the proteinGroups file generated by the MaxQuant analysis. Please note that when running the MaxQuant analysis the option "Requantify" should not be selected at this point. Peptides lacking the light isotope cluster can be considered as fully labeled.
3. The same procedure can also be used for dissociated/fluorescence activated cell sorting (FACS)-sorted planarian cells [12].
4. If the concentration of the protein extract should be out of range of the assay (for the Coomassie (Bradford) Protein Assay (Pierce/Thermo Scientific) between 1 and 1500 μg/ml) we recommend either protein precipitation and subsequent resuspension in a smaller volume to increase sample concentration, or a higher dilution to decrease protein concentration.
5. As the protein pellet is difficult to see, GlycoBlue Coprecipitant (Life Technologies) can be added before the acetone step.
6. Do not overdry the pellet. If the pellet is too dry it will be more difficult to dissolve. It is usually not necessary to further wash the pellet for downstream processes.
7. The Wessel and Fluegge method, which relies on methanol and chloroform [20], can be used as an alternative to the acetone precipitation method described.
8. We recommend the analysis of the H/L ratio of approximately 3 (light) + 3 (heavy) μg of protein (in-solution digestion) prior

to the main experiment. This will allow the adjustment of the mixing ratio prior to protein processing via SDS-PAGE or FASP.

9. To remove residual detergent, the samples can be washed twice with urea buffer. Add urea buffer, briefly incubate and wash through the filter by centrifugation (14,000 rcf for 15 min each).
10. pH indicator paper can be used to control the pH of the solution.
11. For anion exchange chromatography-based fractionation, peptides are resuspended in Britton & Robinson universal buffer pH 11 and loaded on self-packed anion exchange pipette tips containing six layers of 3M Empore disks Anion Exchange material. Anion exchange tips are conditioned by flushing (centrifuge at 7000 rcf) with 100% methanol, 1 M NaOH, and twice with Britton & Robinson universal buffer pH 11. For loading and elution, samples are centrifuged at 800 rcf until the supernatant has passed through completely. Collect the flow-through and elute the remaining peptides with pH buffers 8, 6, 5, 4, 3, 2 each in different tubes or directly onto stage tips. Alternatively, peptides can be separated by offline RP-HPLC at pH 10, combined with concatenated fractionation [21].
12. Coomassie staining solutions or MS-compatible silver staining can be used instead.
13. When the gel is on the glass slide or plastic sheet it can be scanned for documentation. Use a sharp scalpel for cutting the slices. The selection of slice size and number depends on the band pattern on the gel (broad protein bands should not be separated into different slices) and the amount of MS measuring time available. More slices result in better protein separation and therefore in potentially more proteins identified, yet at the cost of increase measuring time.
14. A video tutorial on stage tipping by the authors of [19] is available here: http://www.biochem.mpg.de/226863/Tutorials.

Acknowledgments

We thank S. Zanivan and M. Mann for sharing advice and SILAC mouse liver. This work was supported by the Max Planck Society and the Deutsche Forschungsgemeinschaft (SFB629, PAK479). The Bartscherer lab is part of the Cells-in-Motion Cluster of Excellence (EXC 1003-CiM) and is funded by the European Research Council (ERC-2016-StG 716894 IniReg). We thank J. Rink for helpful comments on the manuscript.

References

1. Sandmann T, Vogg MC, Owlarn S, Boutros M, Bartscherer K (2011) The head-regeneration transcriptome of the planarian Schmidtea mediterranea. Genome Biol 12(8):R76
2. Solana J, Kao D, Mihaylova Y, Jaber-Hijazi F, Malla S, Wilson R, Aboobaker A (2012) Defining the molecular profile of planarian pluripotent stem cells using a combinatorial RNAseq, RNA interference and irradiation approach. Genome Biol 13(3):R19
3. Wenemoser D, Lapan SW, Wilkinson AW, Bell GW, Reddien PW (2012) A molecular wound response program associated with regeneration initiation in planarians. Genes Dev 26(9):988–1002
4. Vogg MC, Owlarn S, Pérez Rico YA, Xie J, Suzuki Y, Gentile L, Wu W, Bartscherer K (2014) Stem cell-dependent formation of a functional anterior regeneration pole in planarians requires Zic and Forkhead transcription factors. Dev Biol 390(2):136–148
5. Vasquez-Doorman C, Petersen CP (2014) zic-1 expression in planarian neoblasts after injury controls anterior pole regeneration. PLoS Genet 10(7):e1004452
6. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Pandey A, Mann M (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 1(5): 376–386
7. Krüger M, Thievessen I, Luber CA, Forner F, Schmidt S, Zanivan S, Fässler R, Mann M (2008) SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. Cell 134(2):353–364
8. Sury MD, Chen JX, Selbach M (2010) The SILAC fly allows for accurate protein quantification in vivo. Mol Cell Proteomics 9(10): 2173–2183
9. Westman-Brinkmalm A, Abramsson A, Pannee J, Gang C, Gustavsson MK, von Otter M, Blennow K, Brinkmalm G, Heumann H, Zetterberg H (2011) SILAC zebrafish for quantitative analysis of protein turnover and tissue regeneration. J Proteome 75(2):425–434
10. Looso M, Borchardt T, Krüger M, Braun T (2010) Advanced identification of proteins in uncharacterized proteomes by pulsed in vivo stable isotope labeling-based mass spectrometry. Mol Cell Proteomics 9(6):1157–1166
11. Larance M, Bailly AP, Pourkarimi E, Hay RT, Buchanan G, Coulthurst S, Xirodimas DP, Gartner A, Lamond AI (2011) Stable-isotope labeling with amino acids in nematodes. Nat Methods 8:849–851
12. Böser A, Drexler HC, Reuter H, Schmitz H, Wu G, Schöler HR, Gentile L, Bartscherer K (2013) SILAC proteomics of planarians identifies Ncoa5 as a conserved component of pluripotent stem cells. Cell Rep 5(4):1142–1155
13. Geiger T, Kruger M, Ishihama Y, Mann M (2011) Use of stable isotope labeling by amino acids in cell culture as a spike-in standard in quantitative proteomics. Nat Protoc 6(2): 147–157
14. Zanivan S, Meves A, Behrendt K, Schoof EM, Neilson LJ, Cox J, Tang HR, Kalna G, van Ree JH, van Deursen JM, Trempus CS, Machesky LM, Linding R, Wickström SA, Fässler R, Mann M (2013) In vivo SILAC-based proteomics reveals phosphoproteome changes during mouse skin carcinogenesis. Cell Rep 3(2):552–566
15. Thomas SN, Wan Y, Liao Z, Hanson PI, Yang AJ (2011) A SILAC-based mass spectrometry approach to detect transient protein interactions using substrate trapping. Anal Chem 83(14):5511–5518
16. 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(12):1367–1372
17. Hubner NC, Mann M (2011) Extracting gene function from protein-protein interactions using Quantitative BAC InteraCtomics (QUBIC). Methods 53(4):453–459
18. Wiśniewski JR, Zougman A, Mann M (2009) Combination of FASP and StageTip-based fractionation allows in-depth analysis of the hippocampal membrane proteome. J Proteome Res 8(12):5674–5678
19. 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(3):663–670
20. Wessel D, Flügge UI (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 138(1):141–143
21. Wang Y, Yang F, Gritsenko MA, Wang Y, Clauss T, Liu T, Shen Y, Monroe ME, Lopez-Ferrer D, Reno T, Moore RJ, Klemke RL, Camp DG 2nd, Smith RD (2011) Reversed-phase chromatography with multiple fraction concatenation strategy for proteome profiling of human MCF10A cells. Proteomics 11(10):2019–2026