Chapter 23

Identification and Regulation of Multimeric Protein Complexes in Autophagy via SILAC-Based Mass Spectrometry Approaches

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Abstract

Mass spectrometry (MS)-based identification and characterization of protein complexes is becoming a prerequisite for in-depth biochemical analyses of intracellular processes. Here, we describe two state-of-the-art MS-based approaches to characterize protein-protein interactions and multi-protein complexes involved in autophagy in mammalian cells. The combination of affinity purification (AP)-MS, which identifies binary protein-protein interactions, with size-exclusion chromatography (SEC)-protein correlation profiling (PCP), which helps monitor protein complex assemblies, is a powerful tool to acquire a full overview of the interlinkage and regulation of novel multi-protein complexes that might play a role in autophagy.

Key words Macroautophagy, Autophagy, Starvation, SILAC, AP-MS, SEC-PCP-SILAC, Proteomics, Mass spectrometry

1 Introduction

Autophagy is a cellular "waste management" system, responsible for degrading intracellular compounds such as out-of-date organelles to regenerate basic nutrients and maintain cellular homeostasis (for review, *see* [1]). In eukaryotic cells, autophagy is constitutively active at basal levels [2] but is activated in response to metabolic or cellular stresses, including starvation, glucose deprivation, or ammonia accumulation [3]. Autophagy is regarded as a pro-survival response and directly linked to diseases, including cancer and neurodegeneration, but also to aging [3, 4], making it a potential target of choice for combination therapies. Although autophagy is extensively studied, there are still many open questions regarding its regulation. As high-throughput analytical techniques are being constantly developed and improved, increasing numbers of putative autophagy regulators are being identified and a need for powerful methods to characterize molecular interactions

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and functional networks addressing these novel regulators emerges. Here, we explain a two-step, mass spectrometry (MS)-based workflow to identify interactomes of putative novel autophagy regulators and to characterize their dynamic composition upon autophagy induction. These assays are amenable to endogenous proteins or to tagged versions of recombinant proteins of interest (baits) expressed in transgenic mammalian cell lines.

In a first set of experiments, binary protein-protein interactions are characterized by affinity purification-mass spectrometry (AP-MS) [5]. In a second set of experiments, the interlinkage and regulation of all the complex members identified by AP-MS are monitored by size-exclusion chromatography (SEC)-protein correlation profiling (PCP) [6]. Both experiments rely on metabolically labeled cells using stable isotope labeling by amino acids in cell culture (SILAC) to generate accurately quantifiable MS data [7]. In addition, the differentially labeled cells are exposed to stress-inducing conditions to trigger autophagy. By AP-MS, stable and labile interactors of the bait are identified from autophagy-induced SILAC-labeled cells [8]. APs of the bait are performed in two ways: (1) pooling the labeled samples before AP will identify the most stable interactors, whereas (2) pooling samples after AP will help identify more labile interactors. The purified proteins are fractionated by SDS-PAGE, digested to peptides, and analyzed by LC-MS/MS. By SEC-PCP-SILAC, native protein complexes are separated according to their size by SEC. Differentially SILAC-labeled fractions are mixed, proteins are therein digested in-solution, and again resulting peptides are analyzed by LC-MS/MS [9]. SEC elution profiles are recorded, and co-eluting proteins are assumed to form multimeric protein complexes. Altogether, this combination of state-of-the-art methods is ideal for a comprehensive characterization of protein complexes and their regulation in autophagy. The use of SILAC to metabolically label cells enables multiplexing, providing an additional layer of information via the identification of stable/labile and condition-dependent protein-protein interactions. In addition, AP-MS and SEC-PCP-SILAC are complementary. While AP-MS primarily identifies direct, binary protein-protein interactions, SEC-PCP-SILAC characterizes multimeric protein assemblies via co-elution.

2 Materials

2.1 Cell Culture and Harvest

1. These assays are established for mammalian cell lines, especially transgenic cell lines designed to ectopically express a tagged version of the bait protein (*see* **Note 1**). Ideally, a control cell line of the same origin, but not expressing any tagged bait, is used as negative control to monitor background binding to

magnetic beads in AP-MS experiments. We routinely use the MCF7 breast cancer cell line (ATCC #HTB-22).

2. SILAC Dulbecco's Modified Eagle Medium (SILAC-DMEM) deficient in arginine and lysine (Gibco).
3. Stable isotope-labeled amino acids: "medium-heavy" L-arginine-$^{13}C_6$ hydrochloride (Arg6) and L-lysine-4,4,5,5-*d*4 hydrochloride (Lys4); "heavy" L-arginine-$^{13}C_6$, $^{15}N_4$ hydrochloride (Arg10) and L-lysine-$^{13}C_6$, $^{15}N_2$ hydrochloride (Lys8) (all from Euriso-top GmbH, Saarbrücken, Germany). Stock solutions prepared in 1 × PBS: arginine isotopes, 0.4 M (2000×); lysine isotopes, 0.8 M (2000×).
4. Unlabeled amino acids: "light" L-arginine (Sigma-Aldrich), L-lysine (Sigma-Aldrich), and L-proline (Fluka). Stock solutions: L-arginine 0.4 M (2000×), L-lysine 0.8 M (2000×), and L-proline 0.17 M (750×), all in 1 × PBS.
5. Dialyzed Fetal Bovine Serum (dFBS, Gibco).
6. 200 mM L-glutamine (100× stock solution, PAN).
7. Penicillin/streptomycin 100× stock solution (10,000 U/mL and 10 mg/mL, respectively, PAN).
8. Sterile 1× Dulbecco's phosphate buffered saline (1 × DPBS, PAN).
9. Trypsin-EDTA solution (200 mg/L trypsin and 500 mg/L EDTA, PAN).
10. 10 and 15 cm tissue culture plates.
11. Cell scraper (Sarstedt).
12. Filter units, e.g., Filtropur series (Sarstedt).
13. Benchtop refrigerated centrifuge for 15 mL conical tubes, e.g., Eppendorf Centrifuge 5810R.

2.2 Autophagy Induction

1. Control, "untreated" SILAC-DMEM medium prepared as above.
2. Starvation medium: Hank's Balanced Salt Solution (HBSS, PAN) or other autophagy induction reagent (*see* **Note 2**).
3. Sterile 1× Dulbecco's phosphate buffered saline (1 × DPBS, PAN).
4. Concanamycin A stock solution: 20 μM in DMSO (10,000× stock solution) (Sigma-Aldrich)

2.3 AP-MS: Cell Lysis, Affinity Purification, Protein Fractionation, and In-Gel Digestion

1. Modified RIPA buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 1 mM EDTA. Store at 4 °C.
2. cOmplete Protease Inhibitors (Roche), aliquoted and stored at −20 °C.

3. PhosSTOP Phosphatase Inhibitors (Roche), aliquoted and stored at −20 °C.
4. Benchtop refrigerated centrifuge for 1.5 mL reaction tubes, e.g., Eppendorf Centrifuge 5415R.
5. BCA protein quantification kit (Thermo Fisher).
6. Plate reader spectrophotometer (absorbance 562 nm, e.g., BioTek KC4)
7. Magnetic beads for IPs:
   (a) Not coupled to antibodies: protein G- or protein A-coupled Dynabeads (Thermo Fisher).
   (b) Tag-specific beads, pre-coupled to antibodies, e.g., Pierce Anti-HA Magnetic Beads (Thermo Fisher); Anti-FLAG M2 Magnetic Beads (Sigma-Aldrich).
8. *Optional*, *for coupling to protein G/A beads only*: tag-specific antibody, 4–8 μg per AP.
9. Magnet for 2 or 15 mL conical tubes (Thermo Fisher). Rotating wheel for incubation.
10. 6× sample buffer: 0.3 M Tris-HCl pH 6.8, 12% SDS, 40% glycerol, 0.05% bromophenol blue.
11. Dithiothreitol (DTT): 100 mM stock solution in water, stored at −20 °C.
12. Iodoacetamide: 550 mM stock solution in water, stored at −20 °C (Sigma-Aldrich).
13. NuPAGE® Novex 4–12% Bis-Tris gradient gels (Thermo Fisher).
14. MOPS running buffer 20× solution (Thermo Fisher): 50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7.
15. Antioxidants (Thermo Fisher).
16. Colloidal Blue Stain (Thermo Fisher).
17. Scalpel (Braun).
18. Parafilm (Pechiney Plastic Packing).
19. ABC buffer: 100 mM ammonium bicarbonate, pH 7.5 (Sigma-Aldrich).
20. Ethanol (HPLC grade, Wako).
21. Trifluoroacetic acid (TFA), 2% (Sigma-Aldrich, MS grade).
22. Buffer A: 0.5% acetic acid in water.
23. Buffer A*: 3% acetonitrile (ACN) and 0.3% TFA in water.
24. Buffer B: 0.5% acetic acid and 80% ACN (Wako) in water.
25. StageTip material (Empore discs, C18 material from 3M).

26. Modified Sequencing-Grade Trypsin (Promega), 12.5 ng/μL in ABC buffer.
27. SpeedVac Concentrator, e.g., Savant SPD121P (Thermo Fisher).

2.4 SEC-PCP-SILAC: Cell Lysis, Ultracentrifugation, SEC, and In-solution Digestion

1. 1× SEC buffer: 50 mM KCl, 50 mM sodium acetate, pH 7.2.
2. SEC lysis buffer: 1× SEC buffer; 1× protease inhibitors (cOmplete Protease Inhibitors, Roche), 1× phosphatase inhibitors (PhosSTOP Inhibitors, Roche).
3. Rotor for ultracentrifugation (e.g., Rotor S100AT6; Thermo Fisher Scientific).
4. Ultracentrifugation tubes (e.g., 4PC tubes; Hitachi Koki).
5. Ultracentrifuge (e.g., Sorvall Discovery M150 SE; Thermo Fisher Scientific).
6. Molecular weight cutoff filter (100 kDa, Vivaspin 2, Sartorius AG).
7. Iodexanol OptiPrep (Sigma-Aldrich).
8. FPLC (e.g., ÄKTA Purifier 10, GE Healthcare).
9. SEC column 600 × 7.8 mm; resolving power 25,000 plates (BioSep SEC s4000, Phenomenex).
10. Gel Filtration Marker Kit for Protein Molecular Weights 29,000–700,000 Da (Sigma-Aldrich).
11. Sodium deoxycholate (DOC): 10% stock solution in water.
12. DTT: 100 mM stock solution in water, stored at −20 °C.
13. Iodoacetamide: 550 mM stock solution, stored at −20 °C (Sigma-Aldrich).
14. Modified Sequencing-Grade Trypsin (Promega), 12.5 ng/μL in ABC buffer.
15. 50% TFA (Sigma-Aldrich, MS grade).
16. Buffer A: 0.5% acetic acid (Sigma-Aldrich) in water.
17. Buffer B: 0.5% acetic acid and 80% acetonitrile (ACN) (Wako) in water.
18. StageTip material (Empore discs, C18 material from 3M).

2.5 LC-MS/MS Analysis

1. LC-MS/MS system (e.g., Q Exactive Plus Hybrid Quadrupole-Orbitrap, Thermo Fisher).
2. ReproSil-Pur C18 AQ 1.9 μm beads (Dr. Maisch GmbH).
3. SilicaTip emitters (New Objective).

3 Methods

The following methods imply the prior identification of a protein with putative functions in autophagy and the stable ectopic expression of a tagged version of this bait protein in a cell line of choice. Ideally, an isogenic cell line not expressing the tagged bait is used as control. In a first set of experiments, stable and labile interactors of the bait are identified by AP-MS. For this, separate APs versus combined APs are performed in SILAC-labeled cells under autophagy-inducing and control conditions (Fig. 1). Once binary protein-protein interactions of the bait are identified, SEC-PCP-SILAC is performed to characterize multimeric protein complexes and to study protein networking (Fig. 2). The combination of both approaches allows to comprehensively characterize interactomes and to define dynamics and regulation upon autophagy induction.

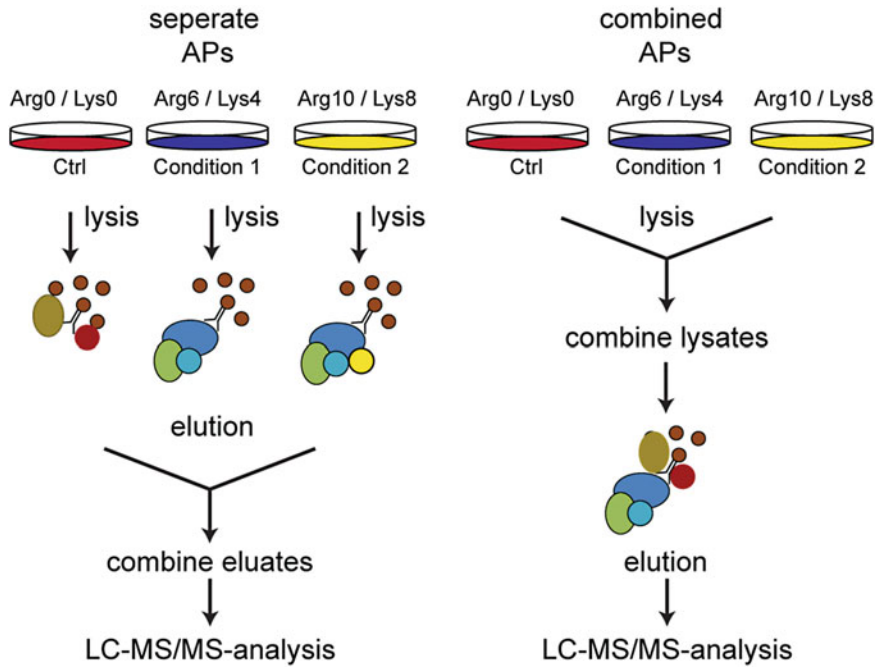

Fig. 1 Affinity purification (AP)-MS for the elucidation of protein-protein interactions. Shown are two SILAC-based approaches that allow the discrimination of (i) stimulus-specific and nonspecific interactions and (ii) of stable and transient interaction partners. Depending on the point of mixing, stable and transient (left) or only stable (right) interactions can be detected. Control samples using cells not expressing the tagged versions of proteins of interest (Ctrl in "light" label) are used to identify unspecific binding proteins. Cells expressing the tagged versions of proteins of interest are treated differently to identify stimulus-specific binding partners (Condition 1 in "medium-heavy" and Condition 2 in "heavy" label). Of note: by AP-MS only binary protein-protein interactions can be studied

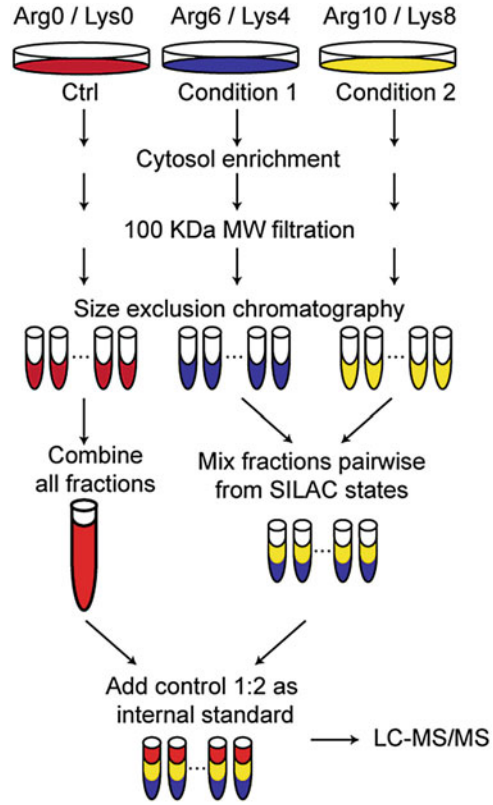

Fig. 2 Size-exclusion chromatography (SEC)-protein correlation profiling (PCP)-SILAC for the elucidation of multimeric protein complexes. Differently SILAC-labeled cells are treated and lysed. The cytosolic fraction is enriched, and multimeric protein complexes are separated by SEC. "Medium-heavy" (Arg6/Lys4) and "heavy" (Arg10/Lys8) labeled fractions are mixed and combined with a "light" labeled internal standard (Arg0/Lys0), which is spiked in a 1:2 ratio into the combined fractions. LC-MS/MS is used to generate elution profiles comparing medium-heavy and heavy to light SILAC ratios, respectively. Co-elution is used as a determinant to define multimeric protein complexes

3.1 Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)

Our experimental setup requires triple SILAC labeling. Commonly a biological replicate with swapped labels is performed in parallel to eliminate isotope-dependent bias. This SILAC labeling method is standard for both the AP-MS and SEC-PCP procedures; however the amounts of cells needed are different. The concentration of SILAC amino acids and proline in the medium is cell line-dependent and needs to be adjusted. This is an exemplified recipe for MCF7 cells.

1. Cells need to be cultured in SILAC medium containing "light" (Lys0 and Arg0), "medium-heavy" (Lys4 and Arg6), or "heavy" (Lys8 and Arg10) labeled amino acids. Prepare these

three SILAC-DMEM media in sufficient volumes for the whole duration of the experiment by dissolving lysine and arginine stocks to 1× concentration, in approximately 10 mL of SILAC medium. Add proline to 1× concentration (*see* **Note 3**), sterile filter the solution, and add it to the final volume of SILAC medium.

2. Complement the filtered SILAC-DMEM with 10% (v/v) dFBS (*see* **Note 4**), 1%(v/v) L-glutamine, and 1% (v/v) Pen/Strep.
3. To gain full incorporation of labeled amino acids, cells should be cultured for at least six cell doublings in SILAC medium [10] (*see* **Note 5**). Incomplete incorporation could lead to quantification inaccuracies. We recommend to start by maintaining each labeling condition in one 10 cm dish and to progressively amplify each population as needed, in order to reach a minimum of two 15 cm dishes per condition per AP, or eight 15 cm dishes per SEC, at the time of autophagy induction.
4. Labeling efficiency and arginine to proline conversion should be analyzed by MS prior to large-scale experiments.

3.2 Autophagy Induction

1. Experimental setup (Fig. 1):
   (a) Control: cell line not expressing the tagged bait, in "light" SILAC-DMEM.
   (b) Condition 1: cell line expressing the tagged bait, in "medium-heavy" SILAC-DMEM.
   (c) Condition 2: cell line expressing the tagged bait, expanded in "heavy" SILAC-DMEM, followed by induction of autophagy, e.g., by full starvation in HBSS for 3 h (*see* **Note 6**).

   To eliminate any putative bias caused by isotope labeling, a biological replicate with swapped labels is also prepared:
   (d) Control: cell line not expressing the tagged bait, in "light" SILAC-DMEM.
   (e) Condition 1: cell line expressing the tagged bait, expanded in "medium-heavy" SILAC-DMEM, followed by induction of autophagy.
   (f) Condition 2: cell line expressing the tagged bait, in "heavy" SILAC-DMEM.
2. Twenty-four hours before treatment, seed the appropriate amount of SILAC-labeled cells to reach 50–70% confluence the next day.
3. On induction day, prepare and warm up the required media. For DMEM-containing media, use the same SILAC-DMEM

(with light, medium-heavy, or heavy amino acids) that each line was maintained in.

4. At T = 0, treat cells with the chosen autophagy induction medium. For starvation conditions, wash cells twice with warm DPBS to remove respective nutrients. Incubate cells in a tissue culture incubator at 37 °C for 1–4 h for early autophagy induction (we routinely use 3 h) or for 7 h for long-term autophagosome accumulation (in the presence of 2 nM concanamycin A).

3.3 Cell Harvest

1. After induction, wash cells twice with ice-cold 1 × PBS in an ice bucket (*see* **Note 7**).
2. Scrape cells in 5 mL 1 × PBS on ice.
3. Pool harvest from all dishes of the same condition in one conical tube, and centrifuge to pellet cells at 1000 × *g* for 5 min at 4 °C.
4. Remove supernatant and immediately freeze pellets in liquid nitrogen (*see* **Note 8**).
5. Store at −80 °C until further use.

3.4 AP-MS

In *separate* APs, each sample is processed independently, and AP eluates of the three differentially labeled conditions are pooled for in-gel MS sample preparation, allowing the identification of both stable and transient interactions. In *combined* APs, the three differentially labeled conditions are mixed prior to performing one single AP, resulting in identifying the most abundant, hence the most stable, interactions formed with the bait (Fig. 1). Provided enough cells were seeded, the same lysates can be used for both separate and combined APs.

3.4.1 Cell Lysis and Affinity Purification

1. Prepare enough lysis buffer for the whole experiment (lysis, APs, and washes) by adding cOmplete protease inhibitors and PhosSTOP Phosphatase Inhibitors to an appropriate volume of modified RIPA buffer, mix, and keep on ice. This reconstituted lysis buffer can be kept at 4 °C for 24 h.
2. Lyse cells in at least three pellet volumes of lysis buffer. Vortex to resuspend, and incubate on ice for 15–45 min, vortexing every 5 min.
3. Centrifuge at 16,200 × *g* for 10 min at 4 °C in 1.5 mL reactions tubes or at 3,320 × *g* for 10 min at 4 °C in 15 mL conical tubes.
4. Transfer supernatants to fresh and labeled tubes, keep on ice.
5. Proceed to protein quantification using a BCA or another detergent-compatible detection kit in parallel with a BSA standard curve. Use the same amount of protein lysate for each AP.

6. Pre-wash magnetic beads: mix beads and pipet 100 µL slurry per AP into a new 1.5 mL reaction tube (prepare a master mix corresponding to $((N + 0.5)*100)$ µL, where N = total number of APs). For volumes higher than 500 µL bead slurry, split in 2 or more tubes. Wash twice with 1 mL lysis buffer per tube, mix by inverting 5 times, and place tube on magnet for 2 min. Resuspend the pre-washed beads in $((N + 0.5)*100)$ µL lysis buffer, and store on ice until needed.
7. *Optional for Protein-G/A Dynabeads: pre-adsorption of the antibody.* (Do not do if using pre-coupled bead/antibody slurry and proceed directly to **step 8**). Mix $((N + 0.5)*100)$ µL (where N = total number of APs) pre-washed beads with $((N + 0.5)*q)/[c])$ µL (where q = amount of antibody per AP, usually 4–8 µg, and [c] = antibody concentration in µg/µL) of specific antibody, and complete to 500 µL with lysis buffer. Incubate 1–2 h at 4 °C with rotation. Place tube on magnet for 2 min, and then eliminate supernatant. Resuspend the bead/antibody mix in $((N + 0.5)*100)$ µL lysis buffer.
8. *Separate APs:* after quantification, prepare the AP tubes (1.5 mL reaction tubes or 15 mL conical tubes) by pipetting 2–5 mg lysate per AP tube (load the same protein amount in all AP samples) and adjusting all reactions to a standard volume (e.g., 1 or 2 mL or up to 10 mL) with lysis buffer. Add 100 µL of pre-washed and pre-coupled bead/antibody solution to each AP, taking care to carefully resuspend the beads in order to load equal bead amounts in all APs. Incubate all APs for up to 6 h at room temperature on a rotating wheel.
9. *Combined APs:* after quantification, pool equal protein amounts of the three SILAC lysates in one AP tube to reach 6–15 mg total protein content. Adjust volume of replicates with lysis buffer when necessary. Add 300 µL of pre-washed and pre-coupled bead/antibody solution to each combined AP. Incubate AP for up to 6 h at room temperature on a rotating wheel.
10. AP washes and elution. Wash APs three times with 1 mL lysis buffer. Mix by inverting 5 times at each wash. After the third wash, elute APs in 2 pellet volumes of 1× sample buffer containing 10 mM DTT by heating at 75 °C for 10 min on a shaker. Spin, place tubes on magnet for 2 min, and recover the eluates in fresh reaction tubes.
11. *Separate IPs only: pool the three eluates in one tube for each experiment.*
12. Store all eluates at −20 °C or proceed directly to in-gel digest.

3.4.2 AP-MS Sample Preparation: In-Gel Digest

1. Alkylate thiols by incubating all eluates with 5.5 mM iodoacetamide for 30 min at RT in darkness.
2. Separate each protein mixture on a NuPAGE® gel, fix the gel by incubation in 50% methanol/10% acetic acid in deionized water for 10 min, and stain/destain it using Colloidal Blue to assess the separation quality.
3. Cut each gel lane into slices of approximately equal sizes, taking care of cutting major bands resulting from the IPs in single slices. Cut each slice into 1 mm^3 cubes, transfer cubes into a reaction tube, and wash out remaining Colloidal Blue by three alternating 10 min incubations in 100 mM ammonium bicarbonate (ABC) buffer and 100% ethanol.
4. Let cubes swell in 80 µL trypsin solution (12.5 µg/mL 100 mM ABC) per slice for 10–15 min at room temperature, then add a minimal volume of ABC buffer until all gel is covered, and incubate overnight at 37 °C.
5. Stop trypsin activity by adding 50 µL of 2% TFA (or until all gel is covered). Shake gel cubes for 10 min at room temperature. Recover the supernatant and transfer solution to new tube. Wash cubes two times with 100–150 µL ethanol to extract all peptides. Combine supernatants of respective slices after each step.
6. Concentrate the collected peptide solution to less than 50 µL in a vacuum concentrator to remove ethanol, and add 200 µL buffer A.
7. Prepare StageTips [11] for desalting of peptide solutions, and proceed by centrifuging solutions through the discs in the following order: 50 µL buffer B to remove impurities and two times 50 µL buffer A to equilibrate and remove buffer B. Now load the sample, wash one time with 100 µL buffer A, and elute the sample with 50 µL buffer B into a new reaction tube.
8. Concentrate eluates to less than 5 µL to remove acetonitrile and add 10 µL buffer A/A* (75/25). The samples are now ready to be analyzed by LC-MS/MS (*see* **Note 9**).

3.5 SEC-PCP-SILAC

SEC-PCP-SILAC allows the accurate recording of SEC protein elution profiles (Fig. 2). Co-elution of proteins is regarded as a determinant to define multimeric protein complexes. In combination with AP-MS approaches, potentially interacting proteins are defined by AP-MS, whereas their spatial network is analyzed by SEC-PCP-SILAC.

3.5.1 Cell Lysis and SEC

Every step should be performed on ice and/or cold conditions to maintain assembled protein complexes.

1. Cool down all centrifuges to 4 °C.
2. Rinse SEC column with water for 1 min with 1 mL/min with valve in load position.
3. Rinse column with SEC buffer for 1 h with 1 mL/min with valve in inject position.
4. Thaw cell pellet on ice with 1.5 mL SEC lysis buffer added; pipet up/down and/or vortex shortly to resuspend cells.
5. Leave resuspended cells on ice for 10 min to swell.
6. Transfer mixture to a clean dounce homogenizer with a "tight" pestle (always keep the homogenizer on ice).
7. Dounce 250–300 times (about 5 min) to lyse the cells.
8. Transfer lysate to a fresh 15 mL conical tube; keep on ice until further usage.
9. Clear lysate from large debris by centrifugation at 4 °C, 3,220 × *g*, 10 min.
10. Add 1 mL of iodexanol to an ultracentrifugation tube.
11. Gently transfer the supernatant after centrifugation on top of the iodexanol, trying to avoid mixing.
12. Equilibrate weights before ultracentrifugation.
13. Ultracentrifuge at 100,000 × *g* 4 °C 20 min (including time to accelerate/decelerate; actual centrifugation time is 15 min). This step ensures that the cytosol is separated from the remaining organelles and membranes.
14. Transfer the upper phase to a 100 kDa MWCO, trying to avoid withdrawing any iodexanol.
15. Decrease sample volume (about 1.2 mL) by centrifugation at 3220 × *g* at 4 °C, until a final volume of 100–200 μL (depending on the protein concentration, this step may vary in duration).
16. Remove sample from the filter using a gel-loading tip, and transfer into a fresh reaction tube. Store on ice until further use.
17. Load sample to the HPLC and separate by SEC (4°-15 °C, flow rate of 0.5 mL/min).
18. Combine the corresponding "medium-heavy" and "heavy" labeled fractions pairwise (1:1).
19. Combine all "light" fractions, mix, and spike in "medium-heavy/heavy" pairs at a 1:2 ratio (1 volume "light", 2 volumes "medium-heavy/heavy"; *see* **Note 10**).

3.5.2 In-Solution Digestion of SEC Fractions

1. Add to each fraction 1% DOC (v/v) and 10 mM DTT.
2. Incubate 30 min at 75 °C.
3. Cool down samples to room temperature, and then add 5.5 mM iodoacetamide.
4. Incubate for 20 min at room temperature in the dark (*see* **Note 11**).
5. Add 2 μg trypsin (1:50 protein to trypsin), and vortex to mix.
6. Digest overnight at 37 °C.
7. Acidify with 15–30 μL of 50% TFA to precipitate DOC and to inactivate trypsin. Vortex thoroughly (*see* **Note 12**).
8. Centrifuge at maximum speed for 20 min at room temperature.
9. Transfer supernatants to fresh 1.5 mL reaction tubes.
10. Prepare StageTips with four disks of C18 materials [11].
11. Wash StageTips with 100 μL buffer B.
12. Wash with 150 μL buffer A.
13. Load the total volume of one fraction successively on one tip.
14. Wash with 150 μL buffer A.
15. Elute with 50 μL buffer B.
16. Concentrate eluates to less than 5 μL to remove acetonitrile and add 10 μL buffer A/A* (75/25). The samples are now ready to be analyzed by LC-MS/MS (*see* **Note 9**).

3.6 LC-MS/MS Analysis

Fractionate the peptide mixture by a reversed-phase chromatography column (SilicaTip emitters, New Objective; ID 75 μm) filled with C18 material (ReproSil-Pur C18 AQ 1.9 μm, Dr. Maisch GmbH), eluting the peptides directly into a mass spectrometer.

3.7 Data Analysis

For efficient analysis of large amounts of MS raw data with peptide identification and protein quantification, we recommend the freely available program MaxQuant, an integrated suite of algorithms specifically developed for high-resolution, high-accuracy quantitative MS data [12]. To assess the quality of a proteomic experiment and perform in-depth analysis of the data, we recommend to use the freely available software Perseus [13] or Graphical Proteomics Data Explorer (GProX) [14] platforms for comprehensive and integrated analysis and visualization of large proteomics datasets. Reproducibility of the experiment can be visualized by transforming the ratios by logarithm to the basis two and plotting two biological replicates in a scatterplot.

AP-MS data yields binary protein-protein interactions: (1) Comparing conditions 1 and 2 with the bead- control discriminates stimulus/autophagy-specific from stimulus-nonspecific interactions (Fig. 1). (2) Plotting separate versus combined APs (e.g., Log2 ratios of autophagy-induced versus control APs)

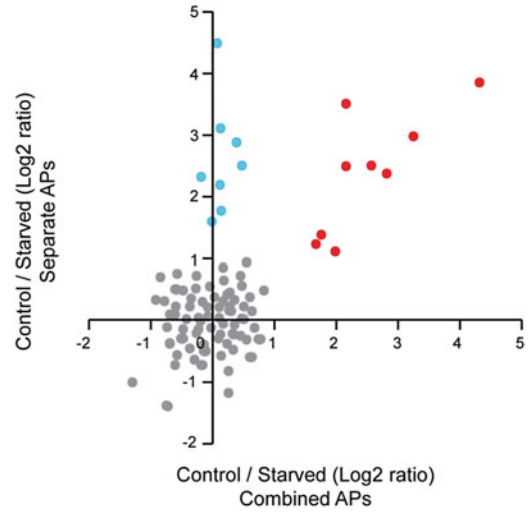

Fig. 3 AP-MS identification of stable and transient interactions (exemplified data). Log2 ratios of starved (Condition 1) versus bead-control protein ratios in combined versus separate APs are plotted. Gray dots: nonspecific interactions when compared to control AP. Red dots: interactors significantly enriched in both combined and separate APs, representing putative stable interactions with the bait. Blue dots: interactors only significantly enriched in separate APs, representing putative labile or transient interactions with the bait. A respective analysis comparing non-starved (Condition 2) versus bead-control protein ratios will identify stimulus-specific interactions

visualizes proteins that are stably enriched with the tagged bait (enriched in both separate and combined APs) and labile interactors (only enriched in separate APs). A theoretical example of respective AP-MS data is shown in Fig. 3. SEC-PCP-SILAC protein ratios are used to visualize co-eluting proteins and to characterize multimeric protein complexes. Figure 4 shows as an example elution profiles of the 14 proteasome core subunits. The 20S and 26S proteasome can be discriminated by overlaying elution profiles of core and accessory subunits (data not shown).

4 Notes

1. Analysis of cell lines expressing tagged versus non-tagged versions of proteins of interest allows the use of the same antibody for purification. This is critical as unspecific binding of background proteins differs between different antibody species. If endogenous proteins shall be analyzed, we recommend to use respective knockdown/knockout cells as control, again using the same antibody for AP [15].

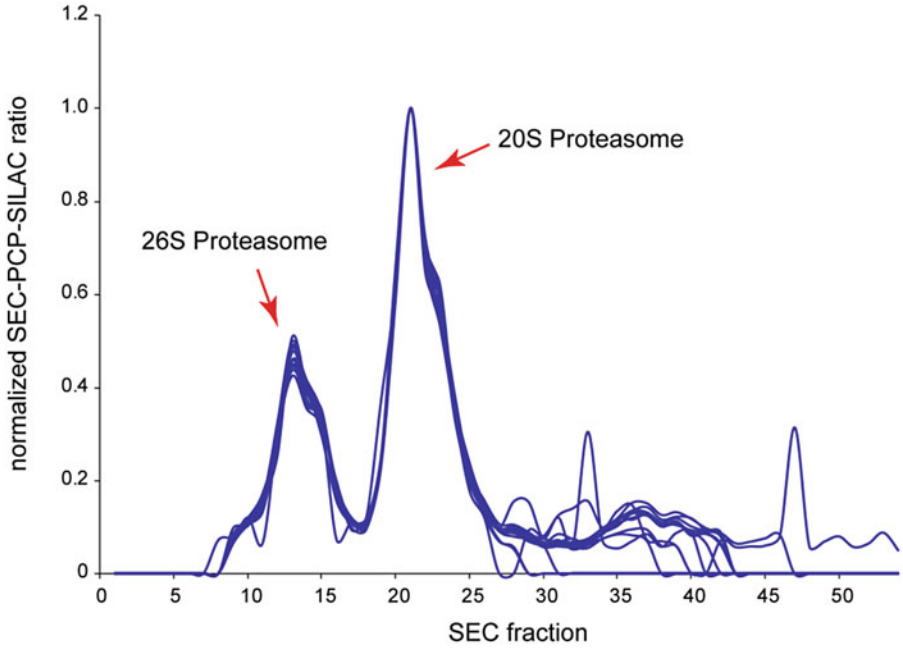

Fig. 4 SEC-PCP-SILAC data characterizing multimeric protein complexes. Shown are normalized SEC-PCP-SILAC ratios of the 14 proteasome core subunits (PSMA1–PSMA7, PSMB1–PSMB7). Two peaks are highlighted characterizing two distinct multimeric protein complexes, the 20S and 26S proteasome. The peak corresponding to the 26S proteasome is shared between core and accessory 19S regulatory subunits (data not shown)

2. Alternatively to full starvation in HBSS, autophagy can be successfully induced with the following reagents:
   (a) HBSS plus dFBS for amino acid starvation.
   (b) Rapamycin treatment: stock solution 100 μM in DMSO (1000×) (Sigma-Aldrich), aliquot, and store at −20 °C.
   (c) SILAC-DMEM without glucose.
3. Unlabeled proline can be added to the medium to avoid metabolic conversion of heavy arginine to heavy proline, which leads to quantification inaccuracies. Arginine and proline concentrations have to be adjusted for each cell line to ensure optimal labeling efficiency.
4. Dialyzed serum has to be used to ensure that only labeled variants of arginine and lysine are present.
5. In saturation-labeling experiments as described here, too short labeling periods result in incomplete label incorporation, which leads to quantification inaccuracies. Next to saturation labeling, dynamic pulse SILAC approaches exist, which allow the determination of protein synthesis, degradation, and turnover [16].

6. If protein complexes are studied that are potentially degraded by autophagy, we recommend to block lysosomal degradation by the addition of 2 nM concanamycin A [2].
7. Complete starvation in HBSS: cells have already been washed and are in serum-free medium. Hence, they do not have to be washed again. This might increase cell yield.
8. If possible, we recommend not freezing cells and directly continuing with AP or SEC as freezing might lead to loss of protein-protein interactions. If samples have to be frozen, this should be done at the stage of cell pellets. Cell lysates should not be stored and should be processed as quickly as possible.
9. If sample dries out while vacuum centrifuging, dissolve peptides with 20 µL buffer B and repeat steps: concentrate eluates to less than 5 µL to remove acetonitrile, and add 10 µL buffer A/A* (75/25).
10. To account for pipetting inaccuracies, increase the volume of the spike in standard by 2 mL of SEC buffer.

    This will ensure that the same volume can be added to all SEC fractions.
11. After IAA incubation, the solution becomes very viscous; this is not a problem for the subsequent steps.
12. Viscosity disappears as soon as TFA is added. Prior to acidification, it might help to freeze samples at −80 °C to help precipitate DOC.

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