Chapter 2

Approaches to Characterize Organelle, Compartment, or Structure Purity

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Abstract

The function of subcellular structures is defined by their specific sets of proteins, making subcellular protein localization one of the most important topics in organelle research. To date, many organelle proteomics workflows involve the (partial) purification of the desired subcellular structure and the subsequent analysis of the proteome using tandem mass spectrometry (MS/MS). This chapter gives an overview of the methods that have been used to assay the purity and enrichment of subcellular structures, with an emphasis on quantitative proteomics using differently enriched subcellular fractions. We introduce large-scale-based criteria for assignment of proteins to subcellular structures and describe in detail the use of ^{15}N metabolic labeling in moss to characterize plastid and mitochondrial proteomes.

Key words Metabolic labeling, Quantitative proteomics, *Physcomitrella patens*, Mitochondria, Plastid, Compartment marker, Density gradient purification

1 Introduction

1.1 Subcellular Protein Localization: Important Though Challenging

Protein sequence information is stored in genes in the nucleus, as well as in the endosymbiont-derived organelles plastids and mitochondria. Yet the majority of effector molecules are proteins, providing catalytic activities and structure to cells. As eukaryotic cells are highly compartmented, subcellular localization of a protein is often of crucial significance to decipher protein and organelle function in the cellular context. With an increasing amount of genome sequence data and of high-quality protein models available for many species, high-throughput studies of the protein composition of subcellular structures become increasingly feasible. Whereas subcellular localization prediction tools can give first hints regarding the putative subcellular localization of proteins, these bioinformatic tools are dependent on the size and quality of training sets and often retain rather high error rates [1, 2].

In addition, cells are highly dynamic and protein compartmentation is subject to variations, both on an evolutionary timescale and

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on a smaller scale between tissues, time of day, and specific environmental conditions [3–5]. The dynamic targeting of proteins to different subcellular localizations has been reported repeatedly and has been termed dual or multiple targeting [3, 6]. Moreover, for several organelles such as ER and Golgi [7], or subcompartments like plastoglobules [8], a constant flux of proteins between compartments is occurring, complicating protein localization studies.

Since the development of various fluorescent protein variants and the availability of custom-made antibodies, the localization of many proteins has been investigated on the single protein level. However, these techniques can imply problems such as artifacts caused by the addition of a protein tag or difficult discrimination between protein isoforms. On the large scale, tandem mass spectrometry (MS/MS) has enabled the study of several hundreds of proteins at the same time, with still increasing sensitivity and dynamic range for complex protein mixtures [9, 10]. In subcellular proteomics datasets, however, the confidence of the assignment of a protein to a certain subcellular structure has become an important issue, due to experimental limitations in the enrichment of subcellular structures.

Arguably, complete purity of a subcellular compartment preparation is barely achievable, as proteins with differing subcellular localizations may co-purify during the isolation process due to several reasons (Fig. 1). These contaminations may be caused by abundant proteins from other subcellular compartments or even represent biologically meaningful information, as "contaminating" proteins may exhibit multiple subcellular localizations or may somehow be associated to the subcellular structure of interest (Fig. 1).

Thus, preparations of subcellular structures should be carefully assessed regarding their purity and proteins only assigned to compartments following in-depth analysis of datasets to avoid misannotation. In recent years quantitative proteomics in particular has contributed powerful workflows to evaluate entire subcellular proteomes.

1.2 Methodological Approaches to Characterize Structure Purity and Protein Localization

After isolation of a subcellular or suborganellar compartment, several aspects of sample quality should be investigated on a regular basis, in order to support the conclusions of a study. This includes assays determining functional integrity, organelle enrichment, and organelle purity. These experiments often reflect facets of the questions: Was the compartment damaged and did it lose proteins during the isolation process? Did the protocol enrich the desired organelle? What amount of contaminating proteins is present in the sample? Several microscopic and biochemical methods were established to answer these questions.

The integrity of organelles can be confirmed by microscopic methods such as light microscopy [1], closer inspection by electron microscopy [8, 11], or biochemical assays for damage such as coupling of mitochondria or the Hill reaction in plastid samples [12].

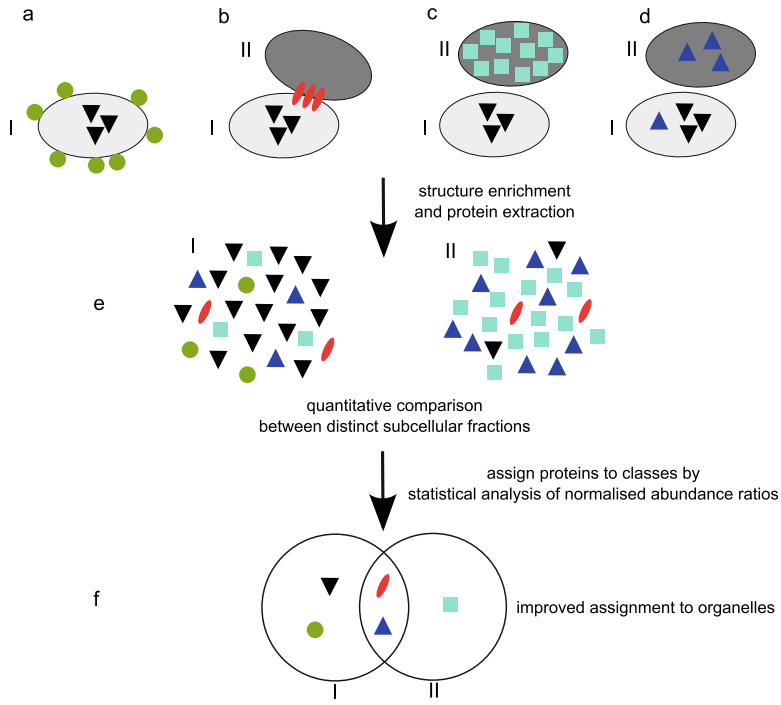

Fig. 1 Generalized examples for cross-contaminating proteins in preparations of subcellular structures. Subcellular structures I and II are membrane-bounded compartments which contain distinct sets of proteins. When subcellular structures are enriched during experimental isolation protocols, several aspects can lead to the co-purification of proteins originating from other subcellular localizations. (**a**) Proteins can be attached to the surface/outer membrane of organelles, such as cytoskeletal proteins or glycolytic enzymes to mitochondria [33]. (**b**) Proteins at organelle interfaces may co-purify with several subcellular structures, such as mitochondria-associated membranes in animals and yeast [34]. (**c**) Abundant proteins from one subcellular structure can be contaminants of preparations of other subcellular structures due to ruptured organelles/organelle fragments (e.g., RuBisCO from chloroplasts). (**d**) Proteins can be truly dually targeted to several organelles, with similar or different relative abundances in the distinct compartments [6, 17]. (**e**) After structure isolation and protein extraction, the protein mixture contains genuine residents of the compartment together with contaminants. (**f**) Quantitative proteomic techniques can assign a relative abundance value to each quantified protein present in different subcellular fractions. These fractions can either be organellar fractions of different purity levels or different purified organelles. Depending on the quantification techniques used, suitable normalization and subsequent statistical analysis should be conducted, resulting in the assignment of proteins to classes with corresponding confidence values. These classes can be attributed to subcellular localizations and improve assignment of proteins to organelles. Note that depending on the experimental design and on the comparisons made, some co-purifying proteins are possibly still not distinguishable from genuine residents (case (**a**))

To check for enrichment, simple tests include light [12] or epi-fluorescence microscopy inspection of the sample using autofluorescence (chlorophyll) or compartment-specific dyes [13]. Biochemically, several approaches are possible, including the quantification of organelle-specific pigments, e.g., for chloroplast envelopes [14], or the detection of organelle-specific marker proteins by either (1) enzymatic activity (*see* Salvi et al. [14] for

tonoplast or plasma membrane, Taylor et al. [12] for mitochondrial markers fumarase and aconitase, chloroplast marker phosphoribulokinase, and peroxisome marker catalase), (2) immunodetection [4, 12], or (3) selected reaction monitoring (SRM) in mass spectrometry [4, 15].

In principle, the same methods are applicable to investigate contaminants from other subcellular fractions in a sample, i.e., the quantification of activity or abundance of compartment-specific markers such as pigments or proteins [1, 13, 14]. Such techniques can give valuable insights into sample quality, though their scope is limited to single proteins that are assumed to be representative for the whole sample. Thus, they are crucial for the initial characterization of a purification protocol and can prove the suitability of the compartment preparation for specific applications, but do not forcibly allow for high-confidence conclusions concerning all proteins in the sample. Moreover, they are dependent on the feasibility of high-purity organelle preparations, which may be problematic for certain organelles (Golgi and ER [7]), tissues, or photoautotrophic growth conditions in plants [16, 17].

Fuelled by the increasing sensitivity of mass spectrometry and the establishment of several quantitative MS/MS techniques, "omics" analyses became applicable to solve this problem and to characterize compartment purity by applying large-scale-based criteria for protein localization in subcellular proteomics experiments. The basic idea behind these techniques is the comparative quantitative analysis of protein abundances, either between different purified subcellular compartments or between differently enriched fractions of the same compartment. Contaminants are often abundant proteins of other organelles which, even after purification of the desired organelles, are still abundant enough to be detected in MS/MS. Consequently these proteins are a lot more abundant in their organelle of origin. Based on this fact, when directly comparing protein quantities in two subcellular fractions, the origin of proteins will be revealed by the protein abundance ratio between the samples under investigation (Fig. 1).

In order to distinguish the origin of proteins in mutual comparisons, different approaches are possible. Labeling with a fluorescent dye was used for enriched vs. depleted mitochondrial fractions using DIGE (difference in-gel electrophoresis or DIGE) with subsequent identification of significantly different protein spots via MS/MS [18]. In quantitative MS/MS, comparative data of two or more protein samples can either be generated in a single MS run, requiring the incorporation of isotope labels into the sample(s), or by comparing protein abundances between different MS runs of unlabeled samples. To date, both strategies have been pursued and yielded convincing results:

The incorporation of iTRAQ (isobaric tags for relative and absolute quantitation) labels into peptides allows for comparisons

between multiple samples and was used for plant cell lysates fractionated on density gradients [7] (termed localization of organelle proteins by isotope tagging, LOPIT), as well as for comparisons between multiple organellar fractions in mouse cells [19]. Similarly, isotope-coded affinity tags (ICAT) were used to label protein samples of yeast peroxisomes [20].

Additionally, isotope labels can be introduced in vivo by either providing isotope-labeled amino acids (SILAC: stable isotope labeling with amino acids in cell culture) or nitrogen salts containing the heavy isotope ^{15}N (metabolic labeling) [21]. To date, the first technique was mainly used for comparisons between different environmental conditions, whereas full metabolic labeling was also applied to mitochondrial and plastid proteomics in moss [17]. For the comparison of unlabeled samples (using several MS runs), spectral counting was successfully employed for plant subcellular fractions. As genuine organellar proteins co-enrich with increasing purity of the corresponding organelle, this abundance trend (also termed quantitative enrichment/quantitative depletion, QE/QD) was used to characterize the mitochondrial proteome [18], the integral membrane mitochondrial proteome [22], and the plastoglobule proteome [8].

After the analysis of quantitative proteomics data, each quantified protein is usually assigned a relative abundance value. As the aim of these methods is to assign proteins to the correct subcellular compartment with a high confidence and to remove contaminants from the "omics" dataset, statistical analyses are necessary to impose thresholds or (un)certainty values concerning the assignment of a protein to the subcellular localization of interest. Multivariate statistics has been used in several variations to achieve this task. Concerning LOPIT and full metabolic labeling, principal component analysis (PCA) with subsequent partial least squares discriminant analysis [7] or clustering [17] has been employed to assign proteins to organelle classes based on their abundance profiles across samples. ICAT ratios were converted into probabilities by statistical modeling of the protein distribution in the investigated subcellular fractions [20]. Following normalization over protein length and total protein amount, threshold ratios [8] or *t*-test [22] was applied to multiple quantitative comparisons using spectral counting.

1.3 Assigning Subcellular Localization by Full Metabolic Labeling in Moss

Full metabolic labeling designates the replacement of all atoms of an element by their respective heavy isotopes in a living organism. The stable nitrogen isotope ^{15}N is present to 0.37% in the atmosphere and can be enriched to about 98% purity in inorganic nitrogen salts (e.g., Cambridge Isotope Laboratories). Plants as photoautotrophic organisms will incorporate such inorganic heavy nitrogen via nitrogen assimilation into all metabolites and proteins. In mass spectrometry, this incorporation will result in a mass shift of 1 u/nitrogen atom and a slightly changed isotope envelope of peptides due to remaining ^{14}N (max. labeling efficiency about 98%, depending on

the purity of inorganic salt used). Metabolic labeling has also been employed in bacteria, fungi, and animals [23], though it is particularly suitable for plants grown in liquid culture or hydroponics [5, 21]. In contrast to the in vivo labeling of plants using isotope-labeled amino acids (SILAC), metabolic labeling can offer advantages regarding experiments involving whole plants or autotrophic conditions and is also suitable for pulse-chase experiments (partial metabolic labeling) [21, 24]. We employed full metabolic labeling with ^{15}N of the model moss *Physcomitrella patens* [25, 26] in order to analyze the mitochondrial and plastid proteomes of the filamentous protonema under photoautotrophic conditions. By relative quantification of protein samples isolated from density-gradient purified "light" mitochondria and "heavy" plastid fractions, and subsequent multivariate statistical analysis employing light/heavy ratios as well as normalized spectral counts, we reliably classified organellar proteins and additionally revealed specific subcellular localizations leading to intermediate light/heavy ratios [17].

2 Materials

2.1 Plant Material, Cultivation, and Metabolic Labeling

1. The *Physcomitrella patens* (Hedw.) Bruch & Schimp wild-type strain (Gransden 2004) is available from the International Moss Stock Center (#40001, IMSC Freiburg, http://www.moss-stock-center.org) [27].
2. An axenic *Physcomitrella patens* protonema culture in mineral Knop medium [28] (250 mg/L KH_2PO_4, 250 mg/L KCl, 250 mg/L $MgSO_4$, 1000 mg/L $Ca(NO_3)_2$, 12.5 mg/L $FeSO_4$, and 10 mL/L of a microelement solution (50 µmol/L H_3BO_3, 50 µmol/L $MnSO_4 \cdot 1H_2O$, 15 µmol/L $ZnSO_4 \cdot 7H_2O$, 2.5 µmol/L KI, 0.5 µmol/L $Na_2MoO_4 \cdot 2H_2O$, 0.05 µmol/L $CuSO_4 \cdot 5H_2O$, 0.05 µmol/L $CoCl_2 \cdot 6H_2O$, pH5.8)), disrupted weekly by an Ultra-turrax (IKA) at 18,000 rpm for 90 s [28].
3. Heavy nitrogen-labeled calcium nitrate ($Ca(^{15}NO_3)_2$). The purity of the ^{15}N should be at least 98 %.
4. 5 L round-bottom flasks with aeration ("bubble flasks") of 0.3 volume of air per volume of medium and minute (vvm) (*see* **Note 1**).
5. Agar plates (1.2 % (w/v)) as controls for axenic moss cultures: Knop medium with 1 % (w/v) glucose, LB medium.

2.2 Isolation of Plastids and Mitochondria from Moss

1. Organelle isolation buffer (OIB) [29]: 300 mM D-sorbitol, 50 mM HEPES-KOH pH 7.5, 2 mM Na-EDTA, 1 mM $MgCl_2$, 0.1 % (w/v) BSA (optional, *see* **Note 2**), 1 % (w/v) polyvinylpolypyrrolidone (PVPP), 0.1 % (v/v) protease inhibitor of choice, such as Sigma Plant Protease Inhibitor Cocktail P 9599.

2. Resuspension buffer (RB): 300 mM D-sorbitol, 50 mM HEPES-KOH pH 7.5, 2 mM Na-EDTA, 1 mM $MgCl_2$ and 0.1% (w/v) BSA (optional), 0.1% (v/v) protease inhibitor of choice.
3. Washing buffer (WB): 300 mM D-sorbitol, 50 mM HEPES-KOH pH 7.5, 2 mM Na-EDTA, 1 mM $MgCl_2$, 0.1% (v/v) protease inhibitor of choice.
4. Household vegetable chopping device (*see* **Note 3**).
5. Cell culture sieve with 100 μm mesh.
6. Büchner funnel, filter, and vacuum pump.
7. Miracloth (Calbiochem).
8. Funnels (fitting centrifugation tubes).
9. Several fine artists paint brushes.
10. Percoll.
11. 50 mL centrifugation tubes.
12. Potter-Elvehjem homogenizer.
13. Pasteur pipettes.
14. Centrifuge, such as Beckman Avanti J-25, with fixed angle rotor for 50 mL tubes, such as JA25.50, capable of 18,000 × *g*, and with adjustable acceleration/deceleration.

2.3 Protein Extraction and Quantification

1. Protein extraction buffer (PEB) freshly supplemented with protease inhibitor of choice, such as Sigma Plant Protease Inhibitor Cocktail P 9599: 7.5 M urea, 2.5 M thiourea, 12.5% (v/v) glycerol, 62.5 mM Tris–HCl, pH 7.8–8.2, 2.5% (w/v) n-octylglycopyranosid, 0.1% (v/v) protease inhibitor.
2. Methanol, chloroform, and bidistilled water for the protein precipitation.
3. Protein resuspension buffer (PRB) for precipitated proteins: 50 mM Tris–HCl pH 8.5, 8 M urea.
4. Chemicals for the protein quantitation assay of choice (*see* **Note 4**).
5. Tris(2-carboxyethyl)phosphine (TCEP) for protein disulfide reduction.
6. 1 M stock solution of iodoacetamide.
7. Keratin-free SDS gels for SDS-PAGE.
8. Coomassie staining solution.
9. Acetonitrile HPLC grade.
10. 100 mM ammonium bicarbonate buffer (ABC).
11. Destaining buffer (DB): 70% (v/v) 100 mM ammonium bicarbonate and 30% (v/v) acetonitrile.
12. 5% (v/v) formic acid (elution additive for LC-MS).
13. Trypsin, MS grade.

2.4 Tandem MS

1. Acetonitrile HPLC grade.
2. Ultrapure water (>18 MΩ).
3. Formic acid (FA) (elution additive for LC-MS).
4. LC-MS setup (*see* **Note 5**).
5. Data analysis software: Mascot Distiller (at least version 2.4) and Mascot Server (at least version 2.2).

3 Methods

3.1 Metabolic Labeling of Moss in Liquid Culture

1. Start a pre-culture at least 5 weeks before scheduled inoculation of the 5 L bubble flasks used for organelle isolation *(see* **Note 6***)*.
2. Prepare parallel cultures: one culture containing only light nitrogen ($Ca(^{14}NO_3)_2$) and one culture containing only heavy nitrogen ($Ca(^{15}NO_3)_2$).
3. Inoculate both cultures with freshly disrupted protonema from the same pre-culture. Do not use more than 50 mg (fresh weight) of protonema to start each culture. Cultivate at least 5 weeks and disrupt the protonema weekly with an Ultra-turrax to keep the moss plants in the protonemal stage. Change the cultivation medium every week. Check for contaminations in the culture using small agar plates containing Knop medium supplemented with glucose and plates containing LB medium, respectively.
4. After 5 weeks the moss should be labeled almost to 98% with ^{15}N. Incorporation of ^{15}N should be checked by mass spectrometry *(see* **Note 7***)*.
5. Prepare aerated 5 L bubble flask with either light ($Ca(^{14}NO_3)_2$) or heavy ($Ca(^{15}NO_3)_2$) nitrogen containing medium using the respective moss material from the pre-cultures. Inoculate the bubble flasks with the same amount (e.g., 3 g fresh weight) of freshly disrupted protonema. Cultivate for 7–10 days with constant aeration at 25 °C and long day (16 h light, 8 h darkness) conditions *(see* **Note 8***)*.

3.2 Enrichment of Plastids and Mitochondria from Moss Protonema

1. All steps should be performed at 4 °C. Precool 100 mL organelle isolation buffer (OIB) per 5 L culture medium.
2. Both types of cultures (light and heavy nitrogen labeled) should be processed separately to avoid cross-contamination. Ensure that organelle isolation of different samples occurs at the same time of day, preferably in the morning due to lower starch content in plastids.
3. Use protonema harvested from two 5 L bubble flasks. Filter moss, first using a 100 µm cell culture sieve, then apply vacuum for about 30–60 s using a Büchner funnel and a vacuum pump. Determine fresh weight (approximately 20 g is required).

4. Subdivide the protonema harvested from one 5 L bubble flask into two fractions. Chop each fraction in approximately 30 mL OIB using the chopping device (50–100 strokes).
5. Carefully decant excess liquid after every 20 strokes. Filter sample through three layers of Miracloth tissue into a 50 mL centrifugation tube using a funnel. After chopping transfer all material onto the Miracloth and wash vessel with the remaining 20 mL OIB.
6. Squeeze Miracloth to recover all liquid, containing released organelles.
7. Spin down released plastids and cell debris at 1500 × g at 4 °C for 15 min. Use slow acceleration and deceleration to prevent damage to organelles.
8. Carefully decant and collect the supernatant (use for purification of mitochondria (*see* Subheading 3.2), **step 19**).
9. Use the remaining pellet for purification of plastids.
10. Resuspend each pellet in 2 mL resuspension buffer (RB) using a fine artists paint brush. Carefully combine all plastid samples, using a cut-off 1 mL pipette tip.
11. Prepare two three-step Percoll gradients per 5 L bubble flask.
12. Apply successively 80% (v/v) Percoll in washing buffer (WB), 40% (v/v) Percoll in WB, 10% (v/v) Percoll in WB into a 50 mL centrifugation tube. Use 5 mL for each Percoll layer. Avoid mixing of the different Percoll layers. If clear interfaces between the different densities are visible, the gradients are ready to use. If even slight mixing is observable, discard the gradient.
13. Carefully layer the organelle suspension (*see* Subheading 3.2, **step 10**) equally on top of all Percoll gradients.
14. Centrifuge the Percoll gradients at 16,000 × *g* at 4 °C for 30 min. Use slow acceleration and deceleration.
15. Transfer the interface between the 80% and the 40% Percoll layer that contains intact chloroplasts using a Pasteur pipette into a fresh 50 mL centrifugation tube.
16. Apply three volumes of washing buffer (WB) and centrifuge at 1500 × *g* at 4 °C for 10 min. Carefully discard the supernatant.
17. Repeat Subheading 3.2, **step 16**. The resulting pellet contains highly pure and intact plastids but also a high amount of insoluble PVPP which does not disturb further processing.
18. Store plastid pellets at −80 °C until further usage.
19. Proceed with the supernatant from Subheading 3.2, **step 8** to purify mitochondria (*see* **Note 9**).
20. Perform two successive centrifugation steps of 3000 × *g* and 6000 × *g*, respectively, in the same tube, each for 5 min at 4 °C to remove nuclei, plastids, and further cell debris.

21. Carefully decant the supernatant into a fresh tube and centrifuge at 18,000 × *g* for 20 min at 4 °C. Use slow deceleration. The resulting pellet is enriched in mitochondria.
22. Carefully decant and discard the supernatant.
23. Resuspend the pellet from each tube carefully in 1 mL WB, using an artist's paint brush (*see* **Note 10**).
24. Combine all resuspended samples in a Potter-Elvehjem homogenizer, adjust the volume to 4.8 mL, and apply ten gentle strokes to completely homogenize the sample.
25. Add 100% Percoll to a final concentration of 20% (v/v) to the resuspended sample (1.2 mL).
26. Prepare a Percoll gradient (one gradient for two 5 L bubble flasks) composed of one layer 80% (v/v) Percoll in WB and one layer 33% (v/v) Percoll in WB (5 mL for each layer). Avoid mixing of the layers (*see* Subheading 3.2, **step 12**). Apply the resuspended pellets in 20% (v/v) Percoll on top of the gradient using a cut 1 mL pipette tip.
27. Centrifuge the gradient at 18,000 × *g* for 1 h at 4 °C. Use slow acceleration and deceleration.
28. After centrifugation the layer containing the mitochondria is visible (a whitish band just above the 80% Percoll interface), a second band of mitochondria is often visible just below the band containing the chloroplasts at the 33%/20% interface (denoted as M1 and M2, *see* Lang et al. [13]).
29. Use a fresh Pasteur pipette to recover the mitochondrial bands from the gradient into a fresh tube (about 4–5 mL).
30. Add three volumes of WB and centrifuge at 18,000 × *g* for 20 min at 4 °C. Use slow deceleration.
31. Carefully remove about two thirds of the supernatant, and repeat Subheading 3.2, **step 30**.
32. Remove all the supernatant, and store the mitochondria-containing pellets at −80 °C.

3.3 Protein Extraction and Mixing of Protein Samples

1. Prepare protein extracts from biological triplicates.
2. Choose an appropriate volume of protein extraction buffer for each organelle pellet (volume depends on pellet size: typically 1–2 mL for plastid pellets and 0.5 mL for mitochondria pellets).
3. Resuspend the organelle pellets in PEB by pipetting up and down.
4. Transfer the resuspended organelles into 2 mL Safe-Lock tubes and vortex vigorously.
5. Place the tubes in an ultrasonic bath for 10 min (*see* **Note 11**).
6. Centrifuge at 20,000 × *g* at RT for 60 min. Do not cool the centrifuge to avoid crystallization of the urea in the extraction buffer.

7. Carefully transfer the supernatant to a 50 mL Teflon tube.
8. Add three volumes of methanol (*see* **Note 12**).
9. Add one volume of chloroform. Vortex vigorously.
10. Add four volumes of bidistilled water. Vortex vigorously.
11. Centrifuge for 15 min at 20,000 × *g* at 0 °C.
12. The precipitated proteins appear at the interface between the aqueous (upper) and the chloroform phase. Remove and discard as much as possible of the upper phase without destroying the interface.
13. Add four volumes of cold (−20 °C) methanol and mix gently by inverting the tube.
14. Incubate at −20 °C for at least 1 h.
15. Repeat centrifugation (*see* Subheading 3.2, **step 11**).
16. Carefully discard the supernatant and let the protein pellet air-dry for a short time.
17. Store the pellet at −80 °C until required.
18. For measurement of the protein concentration, dissolve the protein pellets in protein resuspension buffer (PRB) (*see* **Note 13**).
19. Determine the exact protein concentration and validate the quantification using a Coomassie-stained test gel.
20. Mix equal amounts (30–50 µg) of light protein sample of one organelle (e.g., mitochondria) and heavy protein sample of the other organelle (e.g., chloroplasts) (*see* **Notes 14** and **15**).
21. Reduce the samples with TCEP for 20 min at 28 °C.
22. Alkylate the cysteine side chains in the sample for 20 min at RT in the dark at a final concentration of 25 µM iodoacetamide.
23. Mix the sample with SDS sample buffer and perform SDS-PAGE.
24. Run all samples from the three biological replicates on the same gel.
25. Stain the gel with Coomassie.
26. Cut the whole gel lane into the desired number of Coomassie-stained protein bands (e.g., 15–20), and perform a tryptic in-gel digest. Take care to cut the slices exactly at the same positions in all biological replicates.
27. Chop every gel slice into small pieces using a scalpel.
28. Destain the chopped gel in 100 µl destaining buffer (DB) for 10 min on a shaker at 1200 rpm.
29. Carefully remove the supernatant.
30. Repeat this destaining step (*see* Subheading 3.2, **step 28** and Subheading 3.2, **step 29**) until all Coomassie is removed.
31. Equilibrate the gel pieces for 10 min in 100 µl ABC on a shaker at 1200 rpm.

32. Carefully remove the supernatant.
33. Shrink the gel pieces in 100 % acetonitrile for 5 min on a shaker at 1200 rpm.
34. Remove all acetonitrile and dry the whitish gel in a vacuum concentrator. At this step the gel pieces can be stored at –20 °C.
35. For trypsin digest, apply 0.1 µg trypsin on each gel slice and fill with 100 mM ABC until the gel is rehydrated and completely covered.
36. Incubate at 37 °C overnight.
37. Carefully transfer the supernatant containing the tryptic peptides into a fresh tube (LC-MS tube).
38. Extract the remaining gel pieces additionally with 5 % (v/v) FA for 30 min on a shaker at 1200 rpm.
39. Carefully extract the supernatant and combine it with the first supernatant (*see* Subheading 3.2, **step 37**).
40. Peptides can now be either directly injected for HPLC-MS analysis or dried again in a vacuum concentrator and stored at –20 °C until MS/MS analysis.

3.4 Measurement of Relative Protein Abundance by Mass Spectrometry

1. Perform MS/MS measurements of biological triplicates on a suitable MS/MS platform (*see* **Note 5**).
2. Perform database search of the acquired spectra to identify proteins from the sample using the latest *P. patens* protein models (cosmoss.org). Additional inclusion of a decoy database (e.g., reversed sequences) and a list of sequences of known contaminants (used proteases, human keratin, etc.) is also advisable.
3. As an option, include the specificity of the used protease (usually: trypsin, cuts C-terminal after K/R).
4. Include as variable modifications: oxidation of methionine (+15.994915 Da), carbamidomethylation of cysteines (+57.021464 Da), pyro-glutamate formation of peptide N-terminal glutamine residues (–17.026549 Da) (*see* **Note 16**).
5. The parent ion mass tolerance as well as the fragment ion mass tolerance is defined by the mass spectrometer used and has to be specified in the database search options. A low parent ion mass tolerance (less than 50 ppm) is advisable.
6. Specify allowed charge states of peptides to 2+ and 3+ and search for monoisotopic masses.
7. A list of suitable tools for ^{15}N quantitation as well as a brief description is given in [21].
8. Extract calculated light/heavy ratios for all identified proteins as well as other quantitative values such as spectral counts from the quantitation results.

3.5 Multivariate Statistical Analysis

Light/heavy ratios as well as spectral counts assigned to every quantified protein can be taken into account to analyze a full metabolic labeling experiment [17]. Principal component analysis (e.g., using R www.r-project.org) is a powerful tool to reduce complexity of the data matrix and to derive the uncorrelated variables which are mainly responsible for the variance in the data set. In a comparative experiment between different subcellular fractions, the visualization of these principal components in a plot will already reveal groups of proteins with similar positioning. To mathematically separate and analyze putative protein clusters of different subcellular origins, clustering algorithms are used (e.g., the R mclust package [30]). This additional analysis will allow inference of the number of present clusters and result in clustering probabilities for each quantified protein, leading to an improved assignment to organelles (Fig. 1).

4 Notes

1. Biomass yields are increased by aeration of the culture. As an alternative technique to bubble flasks, 5–10 L tabletop bioreactors are possible [31].
2. BSA can be added to the organelle isolation buffer and the resuspension buffer to serve as interceptor for released proteases upon disruption of cells in order to prevent degradation of proteins. However, it leads to a high fraction of remaining BSA in the samples and therefore may suppress the signals of low-abundance proteins.
3. The amount and force that should be applied when chopping must be enough to break many cells, but should not break too many organelles. For moss protonema onion choppers have proven useful, but the basic principle is to use several interspaced sharp blades. It is advisable to check the quality of your chopped material in a light microscope: There should be many free organelles and few intact filaments.
4. Not every quantification assay is compatible with the proposed urea buffer but the Bradford assay works very well. As an alternative, NanoDrop measurements can be used (A_{280}) to measure the protein concentration, but for this purpose another resolubilization buffer has to be chosen: Typically buffers containing high amounts of urea are incompatible, whereas buffers containing HEPES and SDS are possible. However, the A_{280} method in general overestimates the protein concentration in the sample. Thus, a test gel to check the concentration is necessary.
5. For mass spectrometry at least the MS^1 level should be high resolution (>10000). We suggest using QTOF instruments or better Orbitrap setups (at least Orbitrap XL). In the case of an Orbitrap instrumentation SDS-PAGE could be substituted by

in-solution digest of the protein samples and subsequent analysis of the peptides without prefractionation by using 50 cm HPLC columns attached to the Orbitrap.

6. When labeled moss is required in a short time, it is recommended to keep a small-scale culture (i.e., 30 mL) growing in medium containing ^{15}N. From this culture larger volumes can be inoculated, allowing for high labeling efficiencies.
7. To check the extent of the ^{15}N incorporation, a simple MS measurement of a tryptic digest of a total protein extract is sufficient. After MS analysis, run the quantitation process using Mascot software and specify the purity of the used ^{15}N in the quantitation options. Mascot calculates a theoretical isotope distribution for every identified peptide that is compared to the observed isotope distribution. If the calculated and the observed isotope distributions correspond well to each other, the ^{15}N incorporation is complete.
8. It is crucial to use cultures that are grown in parallel and have the same age. Note that the yield of organelles (especially plastids) decreases with the age of the culture due to differentiation of the moss into gametophores. Cultivation times of 7–10 days are suggested and can yield a more than threefold increase in biomass.
9. Fast processing is essential; we therefore recommend executing the plastid and mitochondrial isolation in parallel, usually requiring two persons.
10. Use different paint brushes for the enrichment of plastids and mitochondria and for labeled and unlabeled samples to avoid cross-contamination of the samples.
11. Optionally an ultrasonic probe can be used. Take care not to heat the sample. Apply three times 20 s of ultrasound.
12. The ensuing methanol/chloroform precipitation is modified after Wessel and Flügge [32] and particularly suitable for lipid-rich samples.
13. Take care not to heat the sample up to more than 60 °C and not longer than 10 min, as the urea in the buffer may artificially modify amino groups (carbamoylation) of peptides. As an option, carbamoylation can be included as variable modification into the MS/MS database search.
14. If in-solution digest in combination with 50 cm high resolution columns is performed instead of SDS-PAGE, mixing 2–3 μg of each protein sample might be sufficient.
15. Relative quantification using a mixing ratio of 1:1 is particularly interesting in cases where proteins present in both samples are of interest. Peptides with very low or high light/heavy ratios are more difficult to quantify. However, reproducibility of light/heavy ratios between biological replicates was very

high [17]. As additional control, reciprocal switching of the labels can be performed.

16. Additionally, peptide N-terminal acetylation (+42.010565 Da) may be included to increase the identification rate, as many plastid proteins undergo N-terminal acetylation subsequent to the cleavage of the transit peptide. However, this option requires specifying the protease specificity to semi-trypsin (only one terminus of the identified peptide needs to represent a tryptic cleavage site, not both). Moreover, artificial modifications of N-termini and lysine side chains by carbamoylation (+43.005814 Da) from the buffer containing urea may be included (*see* **Note 13**). However, addition of these modifications strongly increases the search space and thus requires stringent filtering of the obtained identifications. To increase search speed, carbamidomethylation of cysteines may also be specified as fixed modification.

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