Analysis of Yeast Peroxisomes via Spatial Proteomics

Hirakjyoti Das[1], Alexandros Zografakis[1], Silke Oeljeklaus[1]**, Bettina Warscheid[1,2]*

[1]Biochemistry II, Theodor Boveri-Institute, University of Würzburg, 97074 Würzburg, Germany

[2]CIBSS Centre for Integrative Biological Signalling Studies, University of Freiburg, 79104, Freiburg, Germany.

*corresponding author:

Bettina Warscheid; email: bettina.warscheid@uni-wuerzburg.de

**co-corresponding author:

Silke Oeljeklaus; email: silke.oeljeklaus@uni-wuerzburg.de

Running Head: Spatial Proteomics of Yeast Peroxisomes

Summary

Peroxisomes are ubiquitous organelles with essential functions in numerous cellular processes such as lipid metabolism, detoxification of reactive oxygen species and signaling. Knowledge of the peroxisomal proteome including multi-localized proteins and, most importantly, changes of its composition induced by altering cellular conditions or impaired peroxisome biogenesis and function is of paramount importance for a holistic view on peroxisomes and their diverse functions in a cellular context. In this chapter, we provide a spatial proteomics protocol specifically tailored to the analysis of the peroxisomal proteome of baker's yeast that enables the definition of the peroxisomal proteome under distinct conditions and to monitor dynamic changes of the proteome including the relocation of individual proteins to a different cellular compartment. The protocol comprises subcellular fractionation by differential centrifugation followed by Nycodenz density gradient centrifugation of a crude peroxisomal fraction, quantitative mass spectrometric measurements of subcellular and density gradient fractions and advanced computational data analysis, resulting in the establishment of organellar maps on a global scale.

Key Words: *Saccharomyces cerevisiae*, differential centrifugation, density gradient centrifugation, peroxisome purification, mass spectrometry, label-free quantification, protein localization, spatial proteomics, organellar mapping.

1. Introduction

A defining feature of eukaryotic cells is the compartmentalization into distinct organelles, which enables the partitioning of biological process into diverse subcellular niches that provide specific physiological environments for the different processes. The functionality of cells depends on the controlled transport of proteins, metabolites and signaling molecules between the various subcellular niches, which altogether form a complex, interconnected cellular network. Within this network, peroxisomes fulfil crucial functions in cellular metabolism (e.g., lipid and carbohydrate metabolism), detoxification of reactive oxygen species, and intracellular signaling processes [1–4]. Furthermore, peroxisomes are dynamic organelles that adapt their protein content in response to varying cellular needs [3, 5]. Their importance for health and disease is demonstrated by the occurrence of severe, often lethal diseases caused by defects in peroxisome biogenesis or in the function of individual peroxisomal enzymes [6]. For a comprehensive understanding of peroxisome biology and their different roles within the subcellular network it is in order to define the complete repertoire of peroxisomal proteins, including proteins with dual or multiple localizations, and to monitor alterations in the peroxisomal proteome induced, e.g., by changes in environmental and metabolic conditions or non-functional peroxisomal biogenesis factors. Recent approaches to define the peroxisomal proteome on a global scale made use of (i) bioinformatics prediction and machine learning based on the consensus sequences for the peroxisomal targeting signals (PTS) type 1 and 2, which target proteins to the peroxisomal matrix [7–10], (ii) genome-wide tagging of proteins with a fluorescence tag followed by high-content imaging [11], or (iii) mass spectrometry (MS)-based analysis of subcellular fractions enriched in peroxisomes or peroxisomal membranes obtained by biochemical methods [12, 13]. Studies employing these approaches have greatly contributed to the current knowledge of the peroxisomal proteome of various species. However, there are limitations inherent to each individual approach. Computational approaches 'looking for' PTS1 and PTS2 sequences will miss membrane proteins as well as matrix proteins imported via alternative,

PTS-independent routes such as piggy-backing [14, 15]. Furthermore, the PTS sequences predicted, although present in a protein's amino acid sequence, may not be functional or accessible in the mature folded protein, which would then yield false positives. In case of imaging, the addition of a fluorescence tag such as the green fluorescent protein (GFP) can alter the protein's properties, which may result in its mislocalization. Thus, there is a risk of falsely assigning proteins to peroxisomes or of preventing their peroxisomal assignment.

In MS-based approaches, peroxisomes are typically purified by differential centrifugation and density gradient centrifugation and/or affinity enrichment methods. However, because peroxisomes are small, generally of low abundance, and form physical contact sites with neighboring organelles [16], they cannot be purified to homogeneity. Thus, in MS studies further evidence is needed to discriminate between true peroxisomal proteins and co-purified contaminants. To address this issue, quantitative MS techniques have been applied to accurately measure protein abundances in peroxisomal fractions of different purity, which allows for the identification of *bona fide* peroxisomal proteins based on enrichment factors or intensity profiles [17–21]. Yet, such peroxisome-centered studies generally show only a snapshot of the peroxisomal proteome under a given condition. They do not provide information about dual or multiple subcellular localizations of peroxisomal proteins and they lack information about proteome dynamics, e.g. the relocation of individual proteins to a different cellular compartment in response to changing conditions or the fate of peroxisomal proteins in cells with aberrant peroxisomes. However, such knowledge is indispensable for a holistic view on peroxisome biology.

Advanced MS-based spatial proteomics methodology allows for profiling thousands of native proteins across subcellular compartments [22]. Through quantitative MS analyses of various differential and/or density gradient fractions, proteins are assigned to distinct subcellular niches based on the similarity of their measured distribution profiles. This global approach enables to systematically identify proteins previously not associated with a given compartment as well as multi-localized proteins. Furthermore, the comparison of the protein

distribution profiles established for different conditions or, e.g., in wildtype-*versus*-mutant cell experiments will provide information about proteome dynamics at subcellular resolution.
In this chapter, we describe a spatial proteomics protocol specifically tailored to the study of the peroxisomal proteome of the eukaryotic model organism *Saccharomyces cerevisiae* (Figure 1) including (i) growth of yeast cells using oleic acid as sole carbon source to induce peroxisome proliferation [23], (ii) generation of spheroplasts, (iii) generation of subcellular fractions enriched in nuclei, mitochondria, peroxisomes (i.e. crude peroxisomal fraction), ER/microsomes, and cytosol by differential centrifugation (iv) Nycodenz density gradient centrifugation of the crude peroxisomal fraction, (v) sample preparation, (vi) LC-tandem MS (MS/MS), and (vii) computational analysis and visualization of the spatial proteomics dataset to delineate the peroxisomal proteome in a cellular context. We use label-free quantification for the establishment of protein distribution profiles. However, the protocol can easily be adjusted to the use of alternative MS quantification methods such as isobaric labeling [24–29] or SILAC [25, 30, 31]. Our data analysis pipeline makes use of the programming language R and R packages specifically developed for the analysis of spatial proteomics data. For scientist not proficient in R, we also provide information about alternative tools.

2. Materials

For the preparation of buffers and solutions described below, water of MilliQ purity should be used; reagents and solvents required for LC-MS sample preparation and analysis need to be HPLC grade.

2.1 Yeast and Culture Media

For the protocol described in this chapter, we use *S. cerevisiae* cells grown in oleate-containing medium to induce peroxisome proliferation [23]. Depending on the biological

question being addressed, alternative carbon sources or experiment-specific growth conditions can be used. However, one should be aware that the abundance of peroxisomes may considerably vary under different experimental conditions, affecting the yield of purified peroxisomes.

1. Fresh colonies of the yeast strain of choice, e.g. BY4741 (www.yeastgenome.org/strain/by4741).
2. 20x amino acid mix: L-histidine HCl monohydrate, L-methionine, adenine hemisulfate, uracil, 0.04% (w/v) each; L-tryptophan, L-isoleucine, L-tyrosine, 0.06% (w/v) each; L-arginine HCl, L-lysine, L-phenylalanine, 0.1% (w/v) each; L- leucine, 0.2% (w/v); L-valine, 0.3% (w/v); L-threonine, 0.4% (w/v) (*see* **Note 1**).
3. Synthetic complete (SC) medium: 0.17% (w/v) yeast nitrogen base (YNB) without amino acids, 0.5% (w/v) ammonium sulfate, 0.3% (w/v) glucose, 5% (v/v) 20x amino acid mix, pH 6.0 (adjusted with KOH).
4. Yeast nitrogen oleate (YNO, 5x) medium: 0.85% (w/v) YNB without amino acids, 2.5% (w/v) ammonium sulfate, 25% (v/v) 20x amino acid mix, 0.5% (v/v) oleic acid, 0.25% (v/v) Tween 40, pH 6.0 (adjusted with KOH) (see **Note 2**).
5. Spectrophotometer to measure optical density at 600 nm (OD_{600}).

2.2 Cell Lysis and Preparation of Spheroplasts

1. Dithiothreitol (DTT) buffer (freshly prepared): 100 mM Tris-H_2SO_4 (pH 9.4), 10 mM DTT.
2. Zymolyase buffer (freshly prepared): 1.2 M sorbitol, 20 mM potassium phosphate (pH 7.4).
3. Zymolyase 20-T (MP Biomedicals).
4. Homogenization buffer (pre-cooled at 4°C): 0.6 M sorbitol, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, protease inhibitors (0.3 µM aprotinin, 1 µM bestatin, 5 µM leupeptin, 1.5 µM pepstatin, 1 mM PMSF) (see **Note 3**).
5. Spectrophotometer to measure optical density at 600 nm (OD_{600}).

2.3 Subcellular Fractionation

2.3.1 Differential Centrifugation

1. SEM buffer: 250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2 (adjusted with KOH).
2. 30-mL glass Dounce homogenizer with teflon pestle.
3. Potter homogenizer (e.g., Sartorius Potter S, 150–1,500 rpm/min).
4. Ultracentrifuge (e.g., Optima L-100 XP; Beckman Coulter) with appropriate swinging-bucket rotor (e.g., SW 40 Ti, Beckman Coulter) and ultracentrifuge tubes (14-mL open-top thinwall ultra-clear tubes, Beckman Coulter, product no. 344060) (see **Note 4**).
5. Reagents, protein standard solutions and further equipment to determine protein concentrations, e.g. using the Bradford assay [32].

2.3.2 Nycodenz Density Gradient Centrifugation

1. Nycodenz (Iohexol) density gradient medium (Axis Shield).
2. Nycodenz solubilization buffer (NSB; pre-cooled at 4 °C): 1 mM EDTA, 0.1% (w/v) ethanol, 5 mM MOPS, pH 7.2 (adjusted with KOH).
3. Nycodenz stock solutions (pre-cooled at 4 °C): (A) 30% (w/v; 1.15 g Nycodenz per mL of NSB); (B) 45% (w/v; 1.24 g/mL; (C) 56% (w/v; 1.30 g/ml) (see **Note 5**).
4. 26.3-mL polycarbonate bottles with cap (25 x 89 mm; Beckman Coulter, product no. 355618).
5. Refractometer to measure refractive index of Nycodenz solutions.
6. Peristaltic pump, appropriate tubing (inner diameter of ~1 mm) for the pump, and blunt-ended needle (inner diameter of ~1.25–1.5 mm; length of 10.5 cm).
7. Ultracentrifuge (e.g., Optima L-100 XP; Beckman Coulter) with appropriate fixed-angle rotor (e.g., Type 70Ti, Beckman Coulter).

2.4 LC-MS Sample Preparation

2.4.1 Acetone Precipitation

1. 80% (v/v) acetone, ice-cold.

2. Urea buffer: 8 M urea, dissolved in 50 mM NH_4HCO_3 (ABC), freshly prepared.

2.4.2 Proteolytic In-solution Digestion

1. 50 mM Tris(2-carboxyethyl) phosphine (TCEP), dissolved in H_2O.
2. 500 mM iodoacetamide (IAA), dissolved in 50 mM ABC, freshly prepared.
3. LysC endoproteinase (Promega), dissolved in 50 mM ABC at a final concentration of 150 ng/µL.
4. Trypsin (modified sequencing grade; Promega), dissolved in 20 mM ABC at a final concentration of 75 ng/µL.
5. 100 mM DTT, dissolved in 50 mM ABC, freshly prepared.
6. 2% (v/v) trifluoroacetic acid (TFA).
7. pH indicator paper (low pH range).

2.4.3 Desalting of Peptides

1. 100% methanol (MeOH).
2. Desalting buffer A: 0.5% (v/v) acetic acid (AcOH).
3. Desalting buffer B: 80% (v/v) acetonitrile (ACN)/0.5% (v/v) AcOH.
4. 200-µL pipette tips.
5. Luer lock needle (gauge, G16; outer diameter, 1.7 mm).
6. C18 material (3M Empore or Affinisep).
7. HPLC glass vials with cap (CS-Chromatographie Service GmbH, Germany; article number 300 101), septa (CS-Chromatographie Service GmbH, Germany; article number 300 351) and glass inserts for LC analysis (Macherey-Nagel GmbH & Co. KG, Germany; article number 702 968).
8. 0.1% TFA (v/v).
9. Vacuum concentrator.
10. Ultrasonic water bath.

2.5 LC-MS/MS Analysis

1. Solvent A: 0.1% (v/v) formic acid (FA).
2. Solvent B: 30% (v/v) ACN/50% (v/v) methanol/0.1%(v/v) FA.
3. Nano UHPLC system (e.g., an UltiMate™ 3000 RSLCnano system; Thermo Fisher Scientific), equipped with a C18 pre-column (e.g., nanoEase M/Z Symmetry C18 column; length, 20 mm; inner diameter, 0.18 mm; Waters) and an analytical C18 reversed-phase nano LC column (e.g., nanoEase M/Z HSS C18 T3 column; length, 250 mm; inner diameter, 75 mm; particle size, 1.8 mm; packing density, 100 Å; Waters).
4. ESI-MS instrument (e.g., Orbitrap Elite hybrid mass spectrometer; Thermo Fisher Scientific).

2.6 Data Analysis

1. Software for protein identification, (e.g., MaxQuant/Andromeda, www.maxquant.org [33–35]).
2. Protein sequence database for *S. cerevisiae* (see **Note 6**).
3. Software tools for further data processing and visualization. We recommend to use the statistical programming environment R (https://www.r-project.org) with packages specifically tailored to the analysis of spatial proteomics data such as MSnbase [36] and pRoloc [37] (see **Note 7**). Alternative tools for data analysis are Perseus (https://maxquant.net/perseus/; [38]) and MATLAB https://www.mathworks.com/products/matlab).
4. Lists of organellar marker proteins (e.g., for nucleus, mitochondria, peroxisomes, ER, and cytosol) (see **Note 8**).

3. Methods

3.1 Yeast Cell Culture and Harvest

1. Prepare media for yeast cell culture; sterilize by autoclaving.
2. Per liter of main culture, inoculate 10 mL of SC medium in 100-mL Erlenmeyer flasks with fresh yeast colonies and incubate cultures for approx. 8 h at 30 °C and 160 rpm (first pre-culture).
3. Transfer first pre-cultures into 500-mL Erlenmeyer flasks containing 90 mL of SC medium (second pre-culture). Incubate the cultures overnight at 30 °C and 160 rpm.
4. Measure the OD_{600} (i.e., optical density at 600 nm) of the second pre-cultures.
5. For the main cultures, inoculate SC medium in 5-L Erlenmeyer flasks with cells of the second preliminary culture at an OD_{600} of 0.3 in a final volume of 800 mL. Incubate the cultures for approx. 8 h at 30 °C and 160 rpm.
6. Add 200 mL of 5x YNO to each 800-mL culture. Incubate the cultures overnight at 30 °C and 160 rpm to induce peroxisomal proliferation.
7. Harvest the cells by centrifugation (7,000 x g, 10 min, 4 °C) and resuspend them in deionized water.
8. Combine the cells and wash them twice with deionized water.
9. Determine the wet weight of the cells and immediately proceed with the next steps of this protocol (see **Notes 9** and **10**).

3.2 Cell Lysis and Generation of Spheroplasts

The protocol for cell lysis and the generation of spheroplasts described in the following is based on a protocol published recently [39]. DTT and a mixture of lytic enzymes (i.e., Zymolyase 20-T) are used to remove the cell walls.

1. Resuspend cells in 2 mL of DTT buffer per gram wet cell weight and incubate the cell suspension for 20 min at 160 rpm and 30 °C.

2. Collect the cells by centrifugation (3,000 x g, 5 min, room temperature) and wash them in 7 mL/g wet weight Zymolyase buffer.
3. Collect cells again by centrifugation (3,000 x g, 5 min, room temperature) and resuspend them in 7 mL/g wet weight Zymolyase buffer containing 3 mg/g wet weight Zymolyase 20-T.
4. Measure the OD_{600} of the cell suspension, diluted 1:100 in water. As reference, use a 1:100 dilution of Zymolyase 20-T-containing buffer in water.
5. Incubate for 45 min at 160 rpm and 30 °C.
6. Measure the OD_{600} as described in step 4 to determine the efficiency of cell wall digestion. For sufficient digestion, the OD_{600} after incubation should be ≤ 20% of the value determined before addition of DTT and Zymolyase 20-T [40].
7. Harvest spheroplasts by centrifugation (3,000 x g, 5 min, 4 °C), carefully resuspend them in 7 mL/g wet weight homogenization buffer and immediately proceed with the next steps of this protocol. From this step on, all protein samples need to be kept on ice or at 4 °C at all times.

3.3 Subcellular Fractionation

We here describe a protocol for the preparation of different organellar fractions including nuclear, mitochondrial, peroxisomal, ER/microsomal, and cytosolic fractions obtained by increasing the centrifugal force. During this differential centrifugation, aliquots of the different organelle-enriched fractions will be taken for downstream analysis, e.g. by Western blot or MS analysis (see section 3.3.1) to determine the quality of organelle separation (see **Note 11**). The crude peroxisomal fraction generated by differential centrifugation will subsequently be used for further purification via Nycodenz density gradient centrifugation (see section 3.3.2). Please note that the protocol described in the following has been developed for the enrichment of peroxisomes from BY4741 wildtype cells that were grown under peroxisome-proliferating conditions. The preparation of different organellar (and in particular peroxisome-

enriched) fractions from mutant cells or from cells grown under different conditions may require the adjustment of centrifugal force and/or duration of centrifugation steps.

3.3.1 Preparation of Cellular Fractions by Differential Centrifugation

Transfer spheroplasts to a 30-mL glass Dounce homogenizer.

1. Homogenize spheroplasts using the Potter S (or an equivalent machine) by applying 25 strokes at a speed of 500–600 rpm (see **Note 12**).
2. Transfer the homogenate to a 50-mL tube. Take a 250-µL aliquot of this total cell lysate (referred to as "T") and store it at -20 °C for downstream analysis.
3. Centrifuge the homogenate (1,500 x g, 15 min, 4 °C) to remove nuclei, cell debris and unbroken cells. Transfer the supernatant (S1.5) to a fresh tube. Resuspend the pellet (P1.5, nuclear fraction) in 8–10 mL/g wet weight of SEM buffer and store it at -20 °C.
4. Centrifuge S1.5 as described in step 4, transfer the supernatant (post-nuclear supernatant; PNS) to a fresh tube, take a 250-µL aliquot of the PNS and store it at -20 °C. Discard the small pellet.
5. Prepare a mitochondria-enriched fraction (P5) by centrifugation of the PNS for 20 min at 5,000 x g and 4 °C. Transfer the supernatant (S5) to a fresh tube. Resuspend the pellet (P5) in 2 mL of SEM buffer and store it at -20 °C.
6. Prepare a crude peroxisomal fraction by centrifugation of S5 for 20 min at 13,000 x g and 4 °C. Transfer the supernatant (S13) to a fresh tube and resuspend the pellet (P13) in 2 ml of SEM buffer. Take a 250-µL aliquot of P13 and store it at -20 °C.
7. Centrifuge S13 for 20 min at 17,000 x g and 4 °C. Transfer the supernatant (S17) to a fresh tube, resuspend the pellet (P17; contains ER) in 2 mL of SEM buffer and store it at -20 °C.
8. Prepare cytosolic and microsomal fractions by centrifugation of S17 for 60 min at 100,000 x g and 4 °C in a swinging-bucket rotor. Collect the supernatant (S100, cytosolic fraction), resuspend the pellet (P100, microsomal fraction) in 2 mL of SEM buffer and store both at -20 °C.

9. For further analysis, determine the protein concentration of the following subcellular fractions: whole cell lysate (T), nuclear fraction (P1.5), PNS, mitochondrial fraction (P5), crude peroxisomal fraction (P13), ER/microsomal fractions (P17, P100) and cytosol (S100).
10. Assess the quality of the differential centrifugation and verify the preparation of the crude peroxisomal fraction (P13) by Western blotting (using any standard protocol and antibodies against selected organellar marker proteins, see Table 1) or LC-MS analysis (see section 3.4) of equal amounts of protein per fraction by monitoring the distribution of selected organellar marker proteins across all cellular fractions (see **Note 13**).

3.3.2 Generation of a Gradient-Purified Peroxisomal Fraction by Nycodenz Density Gradient Centrifugation

1. For the generation of Nycodenz density gradients, prepare the following Nycodenz solutions of increasing density from the Nycodenz stock solutions of 30% (A) and 45% (B). For each gradient, mix:
   (1) 1.7 mL of A with 0.3 mL of B,
   (2) 1.4 mL of A with 0.6 ml of B,
   (3) 1.1 mL of A with 0.9 mL of B,
   (4) 0.8 mL of A with 1.2 mL of B,
   (5) 0.6 mL of A with 1.4 mL of B,
   (6) 0.4 mL of A with 1.6 mL of B, and
   (7) 0.2 mL of A with 1.8 mL of B.
   Thoroughly mix the solutions by vortexing.
2. In addition to the Nycodenz solutions prepared in the previous step, the following volumes of stock solutions A, B and C are required for each gradient:
   solution A (30%): 2 mL
   solution B (45%): 3 mL
   solution C (56%): 2 mL

3. Prepare the Nycodenz density gradients using a peristaltic pump (average flow rate of approx. 1 mL/min) and needle, place the tip of the needle at the bottom of the centrifuge tube, and carefully build the gradient starting with 2 mL of 30% Nycodenz solution (i.e. stock solution A, the solution of lowest Nycodenz concentration).
4. Consecutively layer the Nycodenz solutions prepared in step 1 from lowest (#1) to highest (#7) density **under** the previous one. Finish with 3 mL of the 45% Nycodenz solution (i.e. stock solution B) followed by a cushion of 2 mL of 56% Nycodenz solution (i.e. solution C) at the bottom of the tube (see **Note 14**).
5. Freeze the gradients at -20 °C overnight and thaw them 2–3 hours at room temperature prior to use (see **Note 15**). Freeze-thawing of the gradient results in a continuous Nycodenz gradient as shown in Figure 2.
6. Carefully layer the crude peroxisomal fraction (P13; see section 3.3.1, step 6) on top of the Nycodenz gradient by slowly pipetting the sample along the inner walls of the ultracentrifugation tubes.
7. Separate cellular components of the crude peroxisomal fraction by centrifugation for 60 min at 100,000 x g and 4 °C, applying slow acceleration and deceleration rates.
8. Separate the density gradient into 1-mL fractions starting at the bottom (i.e., the fraction of highest density), e.g. using a long needle connected to a peristaltic pump and a fraction collector. Peroxisomal peak fractions are expected to be in fractions 14 and 15 at a density of 1.16–1.19 g/mL. See **Notes 16** and **17**.
9. Determine the protein concentration of gradient fractions #12–#22 (i.e. the fractions of interest for further analysis) using any standard method.

3.4 LC-MS Sample Preparation

We here provide a protocol for the analysis of low protein amounts (starting with 10 µg), which can be applied to assess the quality of organelle separation during differential or density gradient centrifugation, but also to acquire a complete spatial proteomics dataset to generate organellar maps. For deep proteome coverage and to increase the resolution of the

spatial proteomics data, samples may be further fractionated, e.g., by high pH reversed phase chromatography of peptide mixtures [41, 42] obtained from at least 100 µg of protein per fraction. The steps described in the following can easily be adjusted accordingly.

3.4.1 Acetone Precipitation

1. Of the subcellular fractions generated by differential centrifugation (see section 3.3.1, step 10), take aliquots corresponding to 10 µg of protein.
2. Of the gradient fractions #12–#22, take aliquots of equal volume, corresponding to 10 µg of protein in the fraction with the highest protein concentration.
3. Precipitate proteins by addition of the 5-fold volume of ice-cold 80% (v/v) acetone.
4. Incubate samples for at least 2 h at -20 °C.
5. Collect precipitates by centrifugation (12,000 x g, 10 min, 4 °C), remove the supernatant and dry the pellets.
6. Resuspend dried proteins in 10 µL of 8 M urea buffer to reach a final concentration of 1 µg/µL.
7. For better resuspension, incubate samples for 10 min in a water bath sonicator.

3.4.2 Proteolytic In-Solution Digestion

1. Add 1 µL of 50 mM TCEP and incubate samples for 30 min at 37 °C to reduce cysteine residues.
2. Add 1 µL of 500 mM IAA and incubate samples for 30 min at room temperature in the dark to alkylate free thiol groups.
3. Add 3 µL of 100 mM DTT to quench the reaction.
4. Add 50 mM ABC to reach a final urea concentration of 4 M.
5. Add 100 ng of LysC (LysC:protein ratio of 1:100) and incubate samples for 2–4 h at 37 °C.
6. For subsequent tryptic digestion, add 50 mM ABC to reach a final urea concentration of < 2 M.

7. Add 200 ng of trypsin (trypsin:protein ratio of 1:50) and incubate samples overnight at 37 °C or for 4 h at 42 °C.
8. Add approx. 15 µl of 2% TFA to acidify the peptide mixtures and, thus, inactivate the proteases at a pH of 2–3. Check the pH using pH indicator paper.
9. Sonicate all samples for 5 min in a water bath sonicator.
10. Pellet insoluble material (12,000 x g, 5 min, room temperature) and transfer the supernatant containing the proteolytic peptides to a new microcentrifuge tube.

3.4.3 Desalting of Peptides and Preparation of Samples for LC-MS Analysis

For desalting of peptide mixtures obtained from the digestion of low protein amounts, we use self-made StageTips, prepared according to a method described by Rappsilber and colleagues [43] with slight modifications. The StageTips are made of disks of C18 material, each with an estimated binding capacity of 2–4 µg (see **Note 18**).

1. Prepare StageTips by cutting 3 disks of C18 matrix using a Luer lock needle. Gently push the disks into a 200-µL pipette tip using the needle.
2. For each StageTip, prepare a microcentrifuge tube with a hole in the lid.
3. Insert the StageTips into the microcentrifuge tubes through the hole. Make sure that the StageTips are fixed.
4. Add 100 µL of MeOH to activate the StageTips and centrifuge at 800 x g until StageTips are empty.
5. Add 100 µL of Solution B to wash the StageTips and centrifuge at 800 x g until they are empty.
6. Add 100 µL of Solution A to equilibrate the StageTips and centrifuge at 800 x g until they are empty. Repeat this step once.
7. Add the acidified peptide mixtures to the StageTips and centrifuge at 800 x g until empty.
8. Add 100 µL of Solution A for washing and centrifuge at 800 x g until empty. Repeat this step once (see **Note 19**).
9. Place glass inserts into microcentrifuge tubes and transfer the StageTips to the inserts.

10. Add 75 µl of solution B and centrifuge at 800 x g to elute the peptides into the glass inserts.
11. Dry peptides using a vacuum concentrator (see **Note 20**).
12. Reconstitute dried peptides in 45 µL of 0.1% TFA.
13. Incubate samples for 5 min in a water bath sonicator.
14. Collect the reconstituted peptides at the bottom of the glass insert by brief centrifugation, place the inserts into HPLC glass vials, and close the vials with septa and caps.

3.5 LC-MS Analysis

Peptide mixtures are analyzed by high-resolution nano-HPLC-ESI-MS/MS. We here describe parameters for the LC-MS analysis using an Ultimate 3000 RSLCnano system (equipped with pre- and analytical columns as described in section 2.6) coupled to an Orbitrap Elite mass spectrometer.

1. Load an aliquot of the peptide mixtures corresponding to 1 µg of protein onto the pre-column of the RSLCnano system using Solvent A. Wash and preconcentrate peptides for 5 min at a flow rate of 10 µL/min.
2. Switch to the analytical column and elute the peptides applying a flow rate of 300 nL/min and the following gradient: 1–7% solvent B in 5 min, 7–50% B in 245 min, 50–95% solvent B in 85 min, and 5 min at 95% B (see **Note 21**).
3. Operate the Orbitrap Elite with the following parameters: acquisition of MS precursor scans at *m/z* 370–1,700 at a resolution of 120,000 (at *m/z* 400); automatic gain control (AGC) of 1 x 10^6 ions; maximum injection time (IT) of 200 ms; TOP20 method for fragmentation of precursor ions ($z \geq 2$) by low-energy collision-induced dissociation; normalized collision energy of 35%; activation q of 0.25; activation time of 10 ms; AGC for MS/MS scans of 5 x 10^3, maximum IT of 150 ms; dynamic exclusion time of 45 s.

3.6 Data Analysis

Following the protocol described above, we acquired LC-MS/MS data of a total of 19 different subcellular and density gradient fractions per replicate. In the following, we will describe a rudimental workflow to gain a general overview of the obtained spatial proteomics dataset.

1. Use the latest version of the MaxQuant/Andromeda software package (see **Note 22**) for protein identification and label free quantification. Apply MaxQuant default settings with the following exceptions: select Trypsin/P and LysC as enzymes under digestion mode; allow a maximum of 3 missed cleavages; select ‘match between runs’.
2. Set up the software environment R and the MSnbase and pRoloc packages on your computer. Load the libraries of MSnbase and pRoloc for the analysis.
3. Read the MaxQuant results file 'proteinGroups.txt' into R using the read.delim function of R.
4. Convert the proteinGroups dataframe into an ‘MSnSet’ object (see **Note 23**) using the ‘readMSnSet2’ function of the pRoloc package.
5. Once the ‘MSnSet’ object is created, follow the step-by-step guide for data processing and visualization from the tutorial of pRoloc available at the website https://lgatto.github.io/pRoloc/articles/v01-pRoloc-tutorial.html.
6. Save the list of peroxisomal marker proteins (see **Note 8**) as csv or tsv file. Load this list into R and map the entries of the list to 'MSnSet' using the ‘addMarkers’ function in pRoloc.
7. To assess the quality of organelle separation and enrichment of peroxisomes by differential and density gradient centrifugation, generate profile plots (see Figure 1, vii) as described in the pRoloc tutorial to visualize the distribution of the marker proteins of the different subcellular niches across the fractions, using the MS or LFQ intensity as a measure for protein abundance.
8. To visualize the entire spatial proteomics dataset in a single plot, use a dimensionality reduction approach such as the Principal Component Analysis (PCA). This will result in

an overview of the different organellar clusters and further illustrates the separation of the subcellular compartments (see Figure 1, vii). The assignment of proteins to individual clusters allows the identification of new peroxisomal candidate proteins in the peroxisomal cluster. Selected candidates may be validated using orthogonal approaches.

4. Notes

1. The 20x amino acid mix can be frozen and stored at -20 °C.
2. Adjust pH to 6.0 before adding oleate and Tween 40. Mix oleate and Tween 40 well by vigorous shaking before adding the emulsion to the medium. Prepare YNO medium freshly.
3. Add protease inhibitors immediately before use.
4. Alternative instrumentation allowing for the required g-force may be used as well. However, we recommend the use of clear tubes to be able to visually inspect clear separation between pellet and supernatant.
5. To guarantee high reproducibility between experiments, the concentration (in %) or density (in g/mL) of the Nycodenz stock solutions needs to be precise. Determine the refractive index using the refractometer and calculate the concentration or density using the following formulas, provided in the manufacturer's product manual:

   concentration (%, w/v) = (607.75 x refractive index) – 810.13

   density (g/mL) = (3.242 x refractive index) – 3.323.

   If necessary, adjust the concentration/density by adding NSB or Nycodenz to reach the exact values for the Nycodenz solutions A, B, and C.
6. We recommend to download the latest *S. cerevisiae* reference proteome fasta file from UniProt (https://www.uniprot.org/proteomes; proteome ID UP000002311,

Saccharomyces cerevisiae strain ATCC 204508/S288c). As an alternative, a database can be downloaded from the *Saccharomyces* Genome Database SGD (https://downloads.yeastgenome.org/sequence/). However, the data analysis pipeline we describe in this chapter requires the *S. cerevisiae* UniProt identifiers of the proteins.

7. MSnbase [36] is an open-source R-package that provides the infrastructure for processing, annotation, and visualization of proteomics data. For more detailed information, see https://lgatto.github.io/MSnbase/articles/v01-MSnbase-demo.html. pRoloc [37] is a package for the analysis of quantitative MS-based spatial proteomics data that allows to reliably infer the subcellular localization of proteins and that includes, among others, visualization and interactive exploration of the data (see https://bioconductor.org/packages/release/bioc/html/pRoloc.html).
8. To analyze spatial proteomics datasets including, among others, the evaluation of organelle separation by differential and/or density gradient centrifugation as well as the generation of organellar maps, lists of reliable organellar marker proteins are mandatory. Lists of marker proteins for different cellular subcompartments such as ER, mitochondria, Golgi apparatus, cytoplasm, nucleus, and others are provided with the pRoloc package we use for data analysis. However, the package does not contain a list of peroxisomal marker proteins for *S. cerevisiae*. We recommend to use peroxisomal proteins that are known to be exclusively localized to peroxisomes since the inclusion of multi-localized peroxisomal proteins will impair the analysis. This list needs to be based on UniProt identifiers.
9. To guarantee sufficient yield of gradient-purified peroxisomes, we recommend to start with at least 4 g of yeast cells (wet weight). This is an approximate value for wildtype cells grown in oleate-containing medium and may need to be adjusted for the analysis of cells cultivated under different nutritional or stress conditions or in case of mutant cells.
10. To obtain results of high reliability, independent biological replicates of the experiment should be analyzed.

11. For organelle separation of high quality, it is important to obtain a good separation between supernatant and pellet at the individual steps of the differential centrifugation to prevent carry over between soluble and insoluble fraction. Make sure to have a clear interface between pellet and supernatant after each centrifugation step and carefully remove the supernatants using a pipette.
12. The number of strokes for homogenization of the spheroplasts may be reduced, depending on the amount of starting material and how loose/tight the Teflon pestle fits. This is a crucial step, and to ensure reproducibility of the protocol, it should be optimized when setting up the protocol.
13. In case the Western blot or MS data show insufficient separation of different organelles, the g-force and/or centrifugation time at the critical step(s) need to be adjusted.
14. It is very important to place the needle at the bottom of the tube, touching the base, to build the gradient. Make sure not to move the needle until the last Nycodenz solution has been added to the gradient. Care must also be taken when removing the needle. Take it out slowly along the inner wall of the tube to avoid mixing between the Nycodenz solution of different density.
15. Gradients can be stored at -20 °C for several weeks.
16. To assess the reproducibility between different experiments, we recommend to routinely determine the density of each fraction as described in **Note 5**.
17. Before continuing with the computational analysis of the entire LC-MS dataset, we recommend to first assess the quality of the organelle separation by classical Western blotting or LC-MS analysis of the density gradient fractions of interest. According to our experience, these are fractions #12–#22, containing the major subcellular compartments such as nuclei, mitochondria, ER, Golgi, peroxisomes, lysosomes, and cytosol.
18. For desalting of higher peptide amounts, we use C18-SD 7 mm/3 mL extraction disc cartridges (3M Empore) according to the manufacturer's protocol.
19. Peptide mixtures in StageTips can be stored at 4 °C for several weeks. After storage, rehydrate peptides by adding 100 µl of solution A prior to elution.

20. Dried peptides can be stored at -80 °C until the analysis.
21. To assess the quality of organelle separation and the enrichment of peroxisomes, a shorter LC gradient (e.g., of 30–60 min) is sufficient.
22. The MaxQuant/Andromeda software suite is freely available. For information about computational requirements and how to use MaxQuant, refer to the literature [33–35].
23. ‘MSnSet’ is an R data object that contains the quantitative MS data generated in the LC-MS analysis as well as meta data, i.e., information about the design of the experiment, which can either be provided manually or imported as a csv file as described in the tutorial.

5. References

1. Islinger M, Voelkl A, Fahimi HD, Schrader M (2018) The peroxisome: an update on mysteries 2.0. Histochem Cell Biol 150:443–471
2. Kunze M, Pracharoenwattana I, Smith SM, Hartig A (2006) A central role for the peroxisomal membrane in glyoxylate cycle function. Biochim Biophys Acta 1763:1441–1452
3. Smith JJ, Aitchison JD (2013) Peroxisomes take shape. Nat Rev Mol Cell Biol 14:803–817
4. Wanders RJA, Waterham HR (2006) Biochemistry of mammalian peroxisomes revisited. Annu Rev Biochem 75:295–332
5. Schummer A, Maier R, Gabay-Maskit S, et al (2020) Pex14p phosphorylation modulates import of citrate synthase 2 into peroxisomes in Saccharomyces cerevisiae. Front Cell Dev Biol 8:549451
6. Waterham HR, Ferdinandusse S, Wanders RJA (2016) Human disorders of peroxisome metabolism and biogenesis. Biochim Biophys Acta Mol Cell Res 1863:922–933
7. Lingner T, Kataya AR, Antonicelli GE, et al (2011) Identification of novel plant peroxisomal targeting signals by a combination of machine learning methods and in vivo subcellular targeting analyses. Plant Cell 23:1556–1572
8. Emanuelsson O, Elofsson A, von Heijne G, Cristóbal S (2003) In silico prediction of the peroxisomal proteome in fungi, plants and animals. J Mol Biol 330:443–456
9. Nötzel C, Lingner T, Klingenberg H, Thoms S (2016) Identification of new fungal peroxisomal matrix proteins and revision of the PTS1 consensus. Traffic 17:1110–1124
10. Freitag J, Stehlik T, Stiebler AC, Bölker M (2018) The obvious and the hidden: prediction and function of fungal peroxisomal matrix proteins. Subcell Biochem 89:139–155
11. Yifrach E, Holbrook-Smith D, Bürgi J, et al (2021) Systematic multi-level analysis of an organelle proteome reveals new peroxisomal functions. bioRxiv 2021.12.08.471723

12. Schäfer H, Nau K, Sickmann A, et al (2001) Identification of peroxisomal membrane proteins of Saccharomyces cerevisiae by mass spectrometry. Electrophoresis 22:2955–2968
13. Kikuchi M, Hatano N, Yokota S, et al (2004) Proteomic analysis of rat liver peroxisome. Presence of peroxisome-specific isozyme of Lon protease. J Biol Chem 279:421–428
14. Yang X, Edward Purdue P, Lazarow PB (2001) Eci1p uses a PTS1 to enter peroxisomes: either its own or that of a partner, Dci1p. Eur J Cell Biol 80:126–138
15. Effelsberg D, Cruz-Zaragoza LD, Tonillo J, et al (2015) Role of Pex21p for piggyback import of Gpd1p and Pnc1p into peroxisomes of Saccharomyces cerevisiae. J Biol Chem 290:25333–25342
16. Shai N, Schuldiner M, Zalckvar E (2016) No peroxisome is an island - peroxisome contact sites. Biochim Biophys Acta Mol Cell Res 1863:1061–1069
17. Marelli M, Smith JJ, Jung S, et al (2004) Quantitative mass spectrometry reveals a role for the GTPase Rho1p in actin organization on the peroxisome membrane. J Cell Biol 167:1099–1112
18. Islinger M, Lüers GH, Ka WL, et al (2007) Rat liver peroxisomes after fibrate treatment: a survey using quantitative mass spectrometry. J Biol Chem 282:23055–23069
19. Wiese S, Gronemeyer T, Ofman R, et al (2007) Proteomics characterization of mouse kidney peroxisomes by tandem mass spectrometry and protein correlation profiling. Mol Cell Proteomics 6:2045–2057
20. Wiese S, Gronemeyer T, Brites P, et al (2012) Comparative profiling of the peroxisomal proteome of wildtype and Pex7 knockout mice by quantitative mass spectrometry. Int J Mass Spectrom 312:30–40
21. Gronemeyer T, Wiese S, Ofman R, et al (2013) The proteome of human liver peroxisomes: identification of five new peroxisomal constituents by a label-free quantitative proteomics survey. PLoS One 8:e57395
22. Christopher JA, Stadler C, Martin CE, et al (2021) Subcellular proteomics. Nat Rev Methods Primers 1:1–24

23. Gurvitz A, Rottensteiner H (2006) The biochemistry of oleate induction: transcriptional upregulation and peroxisome proliferation. Biochim Biophys Acta 1763:1392–1402
24. Thompson A, Schäfer J, Kuhn K, et al (2003) Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal Chem 75:1895–1904
25. Itzhak DN, Davies C, Tyanova S, et al (2017) A mass spectrometry-based approach for mapping protein subcellular localization reveals the spatial proteome of mouse primary neurons. Cell Rep 20:2706–2718
26. Dunkley TPJ, Watson R, Griffin JL, et al (2004) Localization of organelle proteins by isotope tagging (LOPIT). Mol Cell Proteomics 3:1128–1134
27. Mulvey CM, Breckels LM, Geladaki A, et al (2017) Using hyperLOPIT to perform high-resolution mapping of the spatial proteome. Nat Protoc 12:1110–1135
28. Geladaki A, Kočevar Britovšek N, Breckels LM, et al (2019) Combining LOPIT with differential ultracentrifugation for high-resolution spatial proteomics. Nat Commun 10:1–15
29. Nightingale D, Lilley K, Oliver S (2019) A protocol to map the spatial proteome using hyperLOPIT in Saccharomyces cerevisiae. Bio Protoc 9:e3303
30. Ong SE, Blagoev B, Kratchmarova I, et al (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 1:376–386
31. Itzhak DN, Tyanova S, Cox J, Borner GHH (2016) Global, quantitative and dynamic mapping of protein subcellular localization. Elife 5:1–36
32. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
33. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26:1367–1372

34. Cox J, Neuhauser N, Michalski A, et al (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res 10:1794–1805
35. Tyanova S, Temu T, Cox J (2016) The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat Protoc 11:2301–2319
36. Gatto L, Lilley KS (2012) MSnbase-an R/Bioconductor package for isobaric tagged mass spectrometry data visualization, processing and quantitation. Bioinformatics 28:288–289
37. Breckels LM, Mulvey CM, Lilley KS, Gatto L (2016) A Bioconductor workflow for processing and analysing spatial proteomics data. F1000Res 5:2926
38. Tyanova S, Temu T, Sinitcyn P, et al (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods 13:731–740
39. Morgenstern M, Stiller SB, Lübbert P, et al (2017) Definition of a high-confidence mitochondrial proteome at quantitative scale. Cell Rep 19:2836–2852
40. Cramer J, Effelsberg D, Girzalsky W, Erdmann R (2015) Large-scale purification of peroxisomes for preparative applications. Cold Spring Harb Protoc 2015:db.prot083725
41. Delmotte N, Lasaosa M, Tholey A, et al (2007) Two-dimensional reversed-phase x ion-pair reversed-phase HPLC: an alternative approach to high-resolution peptide separation for shotgun proteome analysis. J Proteome Res 6:4363–4373
42. Morgenstern M, Peikert CD, Lübbert P, et al (2021) Quantitative high-confidence human mitochondrial proteome and its dynamics in cellular context. Cell Metab 33:2464-2483.e18
43. Rappsilber J, Ishihama Y, Mann M (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal Chem 75:663–670
44. Albertini M, Rehling P, Erdmann R, et al (1997) Pex14p, a peroxisomal membrane protein binding both receptors of the two PTS-dependent import pathways. Cell 89:83–92

45. Erdmann R, Kunau WH (1994) Purification and immunolocalization of the peroxisomal 3-oxoacyl-CoA thiolase from Saccharomyces cerevisiae. Yeast 10:1173–1182

Acknowledgment

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 812968 (PERICO). Work included in this study has also been performed in partial fulfillment of the requirements for the doctoral theses of H.D. and A.Z. Furthermore, work in the lab of BW has been supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) Project ID 403222702/SFB 1381 and the Germany's Excellence Strategy (CIBSS – EXC-2189 – Project ID 390939984).

Figures

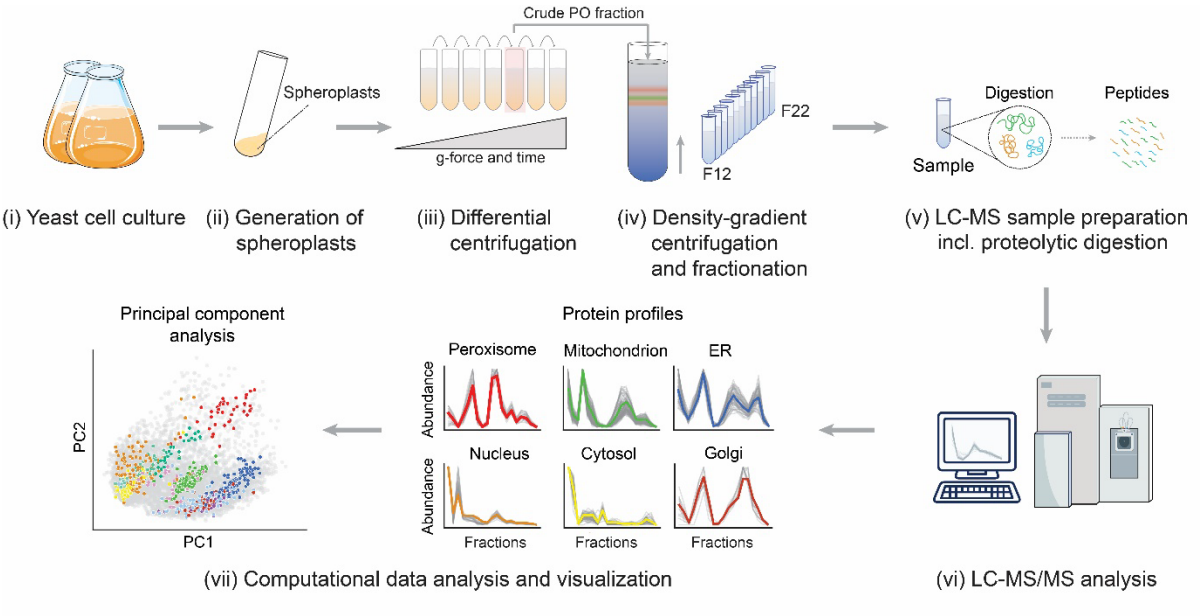

Figure 1. Workflow for the study of the peroxisomal proteome of yeast employing a spatial proteomics approach. For details, see introduction.

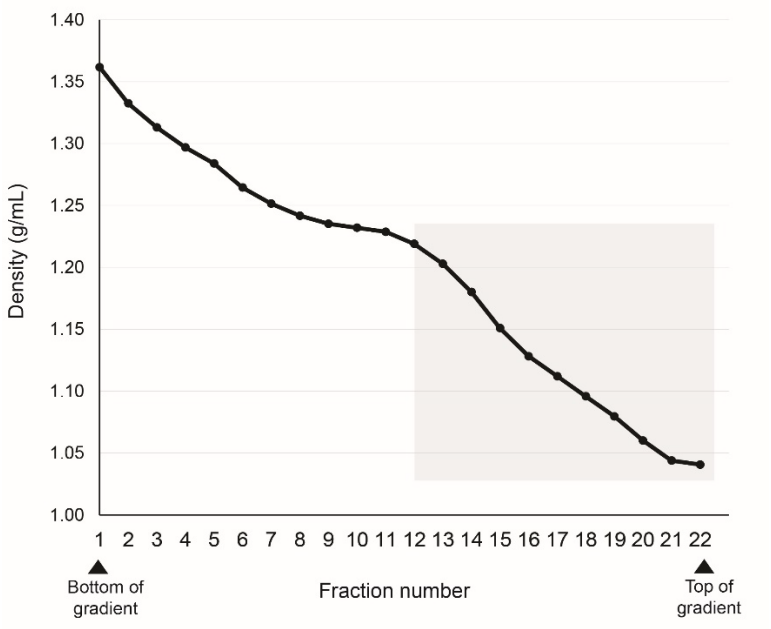

Figure 2. Slope of a Nycodenz density gradient after freeze-thawing. The gradient, generated by layering Nycodenz solutions of different concentrations, was frozen at -20 °C. After thawing the gradient for 3 hours at room temperature, fractions were collected from the bottom (highest density) to the top (lowest density) of the gradient. The refractive index was determined using a refractometer and the density was calculated using the formula provided in **Note 5**. The shaded area marks the fractions of interest, ranging in density from 1.23 to 1.05 g/mL. Peroxisomes of *S. cerevisiae* wildtype cells are expected to be located at a density of 1.16–1.19 g/mL.

Table 1. Examples for antibodies recognizing organellar marker proteins of *S. cerevisiae* in Western blot analyses.

Target Organelle	Primary reactivity and host species	Source	Catalog no.
Peroxisome	rabbit anti-Pex14	Albertini et al., 1997 [44]	-
Peroxisome	rabbit anti-thiolase (Fox3)	Erdmann and Kunau, 1994 [45]	-
Mitochondria	mouse anti-Cox2	Thermo Fisher Scientific	459150
Endoplasmic reticulum	mouse anti-Dpm1	Thermo Fisher Scientific	A-6429
Nucleus	rabbit anti-histone H3	Abcam	ab1791
Cytosol	mouse anti-Pgk1	Abcam	ab113687
Golgi	mouse anti-Pep1	Abcam	ab113690
Plasma membrane	mouse anti-Pma1	Abcam	ab4645