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CHAPTER FOURTEEN

Pexophagy in Hansenula polymorpha

Tim van Zutphen,* Ida J. van der Klei,*† and Jan A. K. W. Kiel*

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Abstract

In the yeast Hansenula polymorpha the development and turnover of peroxisomes is readily achieved by manipulation of the cultivation conditions. The organelles massively develop when the cells are incubated in the presence of methanol as the sole source of carbon and energy. However, they are rapidly and selectively degraded when methanol-grown cells are placed at conditions of repression of methanol metabolism (e.g. in glucose or ethanol excess conditions) by a process termed macropexophagy. Degradation of peroxisomes is also observed when the cells are placed at nitrogen-depletion conditions (microautophagy). This contribution details the methodologies that are currently in use investigating macropexophagy and microautophagy in H. polymorpha. Emphasis is placed on various structural (fluorescence microscopy, electron microscopy) and biochemical (specific enzyme activity measurements, Western blotting) approaches.

* Molecular Cell Biology, University of Groningen, The Netherlands
† Kluyver Centre for Genomics of Industrial Fermentation, Delft, The Netherlands
1. **Introduction**

Organelle homeostasis is a requisite for optimal functioning of eukaryotic cells. One of the modes to achieve this is that specific cell organelles proliferate when required and are removed when they have become superfluous. In eukaryotes, the two major proteolytic degradation processes are the ubiquitin-proteasome pathway and autophagy. The first process is solely involved in the degradation of single protein molecules (Ciechanover, 2006), whereas the latter is capable of degrading a wide range of intracellular constituents (proteins, lipids, or DNA) in the lysosome/vacuole (Klionsky, 2007). Although the process of autophagy was already described by de Duve 45 years ago (de Duve, 1963; de Duve and Wattiaux, 1966), only recently has this topic gained substantial interest, mainly because of its role in a wide variety of processes, such as cell development, ageing, cell death, and immunity (Mizushima et al., 2008). Hence, autophagy is very important in human health and disease (Shintani and Klionsky, 2004).

The key proteins required for autophagy and autophagy related processes are encoded by ATG genes and most are conserved from yeast to man (Meijer et al., 2007). Because of their versatility in handling, yeast species are ideal model organisms for studying the molecular mechanisms of autophagy. Indeed, most ATG genes were initially discovered in yeast (Klionsky et al., 2003). In particular, the process of peroxisome degradation is studied in these organisms because the development and turnover of these organelles is readily manipulated by the growth conditions. Here we describe the degradation of peroxisomes by autophagy in the methylotrophic yeast *Hansenula polymorpha* and focus on the experimental approaches that are used to monitor this process.

2. **H. polymorpha as a Model System for Peroxisome Degradation**

A limited number of microorganisms are capable of growing on one-carbon compounds (methylotrophs). In yeast species, methylotrophy is limited to the utilization of methanol as the sole source of carbon and energy. Examples of methylotrophic yeast species are *Candida boidinii*, *H. polymorpha*, and *Pichia pastoris*. In these organisms the initial oxidation of methanol is catalyzed by the enzyme alcohol oxidase (AO), which is a peroxisomal oxidase that generates formaldehyde and hydrogen peroxide from methanol. Formaldehyde can be assimilated via the xylulose-5-phosphate pathway, which involves the peroxisomal enzyme dihydroxyacetone synthase (DHAS), whereas hydrogen peroxide may be decomposed by
peroxisomal catalase (CAT). The other enzymes involved in methanol metabolism (e.g., formaldehyde dissimilation enzymes and the other enzymes of the xylulose-5-phosphate pathway) are all localized to the cytosol (reviewed in van der Klei et al., 2006).

When methylotrophic yeast species are grown on media containing glucose as the sole carbon and energy source and ammonium sulfate as nitrogen source, peroxisomal enzymes are not required for primary metabolism. As a consequence, the cells generally contain one or only a few small peroxisomes. However, upon a shift to media containing methanol as the sole carbon source, enzymes involved in methanol metabolism are induced concomitant with an increase in the number and size of peroxisomes. Conversely, placing methanol-grown *H. polymorpha* cells into fresh glucose media leads to rapid degradation of the—now superfluous—organelles. This degradation of peroxisomes (pexophagy), is highly selective and resembles macroautophagy in mammalian cells (Leighton et al., 1975) and hence is designated macropexophagy (Klionsky et al., 2007). During this process individual organelles are consecutively sequestered from the cytoplasm by membranous layers forming autophagosomes. After sequestration is complete, the outer membrane layer of the autophagosome fuses with the vacuolar membrane, resulting in incorporation of the sequestered peroxisome into the vacuole, where the entire organelle becomes degraded by vacuolar hydrolases (depicted in Fig. 14.1A). The molecular mechanisms involved in the formation of autophagosomes and fusion of this organelle with the vacuole overlap with those involved in general macroautophagy, because various Atg proteins are involved in both processes.

Initiating macropexophagy, however, requires the function of two peroxisomal membrane proteins, Pex3 and Pex14. The function of these two peroxins, that are also essential for peroxisome biogenesis, is completely different. Remarkably, one of these, Pex3, has to be removed from the peroxisomal membrane to allow initiation of pexophagy, whereas Pex14 probably acts as the site of recognition of the organelle by the autophagy machinery (Bellu et al., 2001a, 2002). Recognition most likely involves Atg11, a protein also functional in other selective modes of autophagy (Kiel et al., 2003). Atg11 has been localized to the PAS (preautophagosomal structure) and autophagosomal membranes and is thought to be involved in recruiting selective cargo to the autophagosome (Yorimitsu and Klionsky, 2005).

In the related methylotrophic yeast *P. pastoris*, a peroxisomal membrane protein exclusively involved in selective peroxisome degradation was recently uncovered (designated Atg30) and may be involved in this process as well (Farre et al., 2008). A putative Atg30 ortholog is present in *H. polymorpha*, but its function in pexophagy has not yet been confirmed.

In addition to macropexophagy, nitrogen limitation leads to peroxisome degradation in *H. polymorpha* by a mechanism known as microautophagy.
However, this process is not considered a selective pathway, as cytosolic components are taken up concomitantly with peroxisomes (Bellu et al., 2001b). Induction of microautophagy by nitrogen starvation results in a direct engulfment and subsequent uptake of peroxisomes and cytoplasmic components by the vacuole (Fig. 14.1B). Microautophagy is therefore morphologically very distinct from macropexophagy. Nevertheless many components of the macroautophagy machinery function also in microautophagy (Sakai et al., 2006). Remarkably, in *H. polymorpha* microautophagic degradation of peroxisomes, but not of other cytoplasmic constituents, requires Atg11, suggesting some mode of selectivity of peroxisome degradation during nitrogen starvation (Komduur et al., 2004).

**Figure 14.1** Methanol-grown *H. polymorpha* cells contain mature peroxisomes (P) as well as at least one immature organelle. (A) Upon induction of macropexophagy, a single mature organelle is tagged for degradation (marked by the black dot) followed by its sequestration by multimembrane layers forming an autophagosome (AP). After sequestration is completed, the outer membrane layer of the autophagosome fuses with the vacuolar membrane resulting in the uptake of the organelle into the vacuole (V), where it is degraded by vacuolar hydrolases. Next, one by one, other mature peroxisomes are tagged and degraded. Only one (or few) immature peroxisome(s) escape(s) degradation. (B) Upon induction of microautophagy the vacuole engulfs (part of) a cluster of peroxisomes that is subsequently degraded in the vacuole lumen.
3. **Cultivation of H. polymorpha and Induction of Pexophagy**

3.1. Cultivation of *H. polymorpha* in media supplemented with methanol as the sole carbon and energy source

For induction of pexophagy, cells from the exponential growth phase on methanol are preferably used. Care should be taken that all nutrients are present in excess and that cultures are optimally aerated.

We generally use (auxotrophic derivatives of) the *H. polymorpha* strain NCYC495 and mutants generated from this strain (Gleeson and Sudbery, 1988). For inoculation of glucose-containing batch cultures, colonies plated on a glucose-containing agar plate (e.g., YPD plate) are used. These plates can be stored at 4 °C for several weeks.

For precultivation, a mineral medium (van Dijken *et al.*, 1976; Table 14.1) is used that contains 0.25% ammonium sulfate as nitrogen source and is supplemented with 0.5% glucose as carbon source. Optimal growth of *H. polymorpha* cells on methanol media and maximal induction of peroxisomes is only obtained when cells are extensively pregrown on

<table>
<thead>
<tr>
<th>Components</th>
<th>g/l</th>
<th>Vishniac stock solution (1000x)</th>
<th>g/l</th>
<th>Vitamin stock solution (1000x)</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>(NH₄)₂SO₄</em></td>
<td>2.5</td>
<td>EDTA (Titriplex-III)</td>
<td>10</td>
<td>Biotin</td>
<td>0.1</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.2</td>
<td>ZnSO₄·7H₂O</td>
<td>4.4</td>
<td>Thiamin</td>
<td>0.2</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.7</td>
<td>MnCl₂·4H₂O</td>
<td>1.01</td>
<td>Riboflavin</td>
<td>0.1</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>3.0</td>
<td>CoCl₂·6H₂O</td>
<td>0.32</td>
<td>Nicotinic acid</td>
<td>5</td>
</tr>
<tr>
<td>yeast extract</td>
<td>0.5</td>
<td>CuSO₄·5H₂O</td>
<td>0.315</td>
<td>p-Aminobenzoic acid</td>
<td>0.3</td>
</tr>
<tr>
<td>Vishniac stock solution</td>
<td>1.0 ml</td>
<td>*(NH₄)₆Mo₇O₂₄·4H₂O</td>
<td>0.22</td>
<td>Pyridoxal hydrochloride</td>
<td>0.1</td>
</tr>
<tr>
<td>Add after autoclaving:</td>
<td></td>
<td>CaCl₂·2H₂O</td>
<td>1.47</td>
<td>Ca-panthenate</td>
<td>2</td>
</tr>
<tr>
<td>vitamin stock solution</td>
<td>1.0 ml</td>
<td>FeSO₄·7H₂O</td>
<td>1.0</td>
<td>Inositol</td>
<td>10</td>
</tr>
<tr>
<td>Carbon source</td>
<td>0.5 %</td>
<td>Sterilize by filtration</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
glucose prior to the shift to methanol medium. Therefore, methanol-grown cells should not be used as inoculum for these cultures.

Protocol

1. Normally, cells from fresh glucose plates are used as inoculum. Cells are precultured at 37 ºC at 200 rpm in a 100-ml flask with 20 ml of mineral medium containing 0.5% glucose until an optical density (OD, expressed as absorption at 660 nm) of 1.5–1.8 is reached.
2. The culture is diluted to OD660 = 0.1 in fresh glucose medium and grown again until the mid-exponential growth phase. This procedure is normally repeated 3 times until the cells continuously grow at maximal speed (doubling time of approximately 1 h for wild-type cells).
3. Cells from the mid-exponential growth phase (OD660 = 1.5–1.8) are diluted in 100 ml of fresh mineral medium containing 0.5% methanol as the sole source of carbon (starting at OD660 = 0.1) in a 500-ml flask. After a short lag phase wild-type cells start to grow on methanol (normal doubling time on methanol is approximately 4–4.5 h). Cultures at the late exponential growth phase (OD660 = 1.8–2.4) are used to induce peroxisome degradation.

3.2. Glucose/ethanol induced macropexophagy

In H. polymorpha, macropexophagy is induced by exposure of methanol-grown cells to excess glucose or ethanol conditions. Both addition of glucose/ethanol to cultures growing on methanol as well as a dilution of methanol-grown cells in fresh glucose/ethanol media have been applied successfully. Although addition of glucose or ethanol to a methanol culture is experimentally easier, it must be noted that prolonged cultivation may ultimately lead to depletion of medium components (e.g., vitamins, amino acids) as cultures with very high densities may be obtained. This carries the risk of induction of nonselective autophagy as a result of starvation as well. Hence, a shift of cells to fresh glucose/ethanol medium is preferred.

Protocol

1. Dilute the methanol culture in fresh, prewarmed medium lacking a carbon source to an OD660 = 0.2.
2. Immediately and rapidly take a sample (T = 0 h; for OD measurement and biochemical or microscopy analysis, see subsequent sections), followed by addition of inducer (glucose, ethanol) to a final concentration of 0.5%.
3. Continue to incubate the cells at 37 ºC at 200 rpm.
4. Take samples of an equal culture volume as performed at step 2, at T = 1, 2, 3 and 4 h after the addition of the inducer.
3.3. N-starvation induced microautophagy

Upon a shift of *H. polymorpha* cells from media containing excess nitrogen (generally ammonium sulfate) to media lacking any nitrogen source (i.e., also excluding amino acids), nonselective autophagy is induced.

**Protocol**

1. Cells are grown on methanol (100 ml), as described previously.
2. Cells are collected by centrifugation (5 min at 3000×*g* at 37 °C).
3. The supernatant fraction is discarded and the cells are rapidly resuspended in an identical volume of prewarmed mineral medium lacking any nitrogen source.
4. Samples are taken at 2-h intervals to follow the fate of peroxisomes, and other proteins, over time.

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### 4. ANALYSIS OF PEROxisome Degradation

To determine whether degradation of peroxisomes has occurred, several approaches are suitable. A combination of at least two experimental approaches is preferred.

#### 4.1. Biochemical analysis

**4.1.1. Preparation of cell extracts for biochemical analysis**

Peroxisome degradation can be demonstrated by Western blot analysis of samples taken prior to, and after, the induction of autophagy, using antibodies against peroxisomal marker proteins.

For Western blot analysis, a culture volume corresponding to at least 3 OD<sub>660</sub> (volume × OD<sub>660</sub>) units is harvested. Three OD<sub>660</sub> units correspond to approximately 300 µg of protein. Upon induction of peroxisome degradation, the cells may continue to grow, while peroxisome formation and the synthesis of peroxisomal proteins of methanol metabolism (AO, DHAS, and CAT) is fully repressed. Thus, equal volumes of the culture before and after induction of peroxisome degradation should be studied.

**Protocol**

1. Cells are precultured in glucose, grown in methanol-containing medium, and then shifted to glucose, ethanol or nitrogen-starvation medium as described in section 3.
2. Cultures are cooled on ice followed by collection of the cells by centrifugation (10,000×*g* for 10 min at 4 °C in case of large volumes or for 1 min at 14,000×*g* in a microcentrifuge when using small volumes).
3. The supernatant fractions are discarded and the cell pellets are resuspended in a solution containing 12.5% trichloroacetic acid (TCA) by vortexing, followed by freezing at $-80^\circ C$ for at least 30 min.

4. For analysis, the frozen samples are thawed on ice and centrifuged at 14,000×g at 4°C for 5 min to collect the cells.

5. The cell pellet is washed twice (by resuspending thoroughly followed by centrifugation) using 500 µl of 80% ice-cold acetone (v/v) to remove residual TCA.

6. The pellet is air-dried and resuspended in 100 µl of a solution containing 1% SDS and 0.1 N NaOH. The high pH of this solution causes the yeast cell wall to disintegrate whereas SDS dissolves the cellular membranes. The pellet is resuspended (using a vortex) until a homogeneous cell suspension is obtained.

7. The suspension is mixed with an equal volume of concentrated SDS sample buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.002% bromophenol blue, 0.1 M Tris–HCl, pH 6.8) and boiled for 5 min.

8. Prior to loading onto an SDS-polyacrylamide gel, the sample is centrifuged for 3 min at 10,000×g to sediment residual cell debris. The gels are used for Western blotting to analyze the levels of peroxisomal marker proteins. Cytosolic and/or mitochondrial marker proteins should be used as controls to monitor whether or not selective peroxisome degradation has occurred.

### 4.1.2. Analysis of pexophagy by Western blot analysis

Because the synthesis of the methanol metabolism-related peroxisomal matrix proteins is fully repressed in glucose or ethanol excess conditions, the biochemical analysis of peroxisome degradation has focused on following the amount and/or specific activities of these proteins. A distinction can be made between peroxisomal matrix proteins and membrane proteins. In specific cases in which matrix proteins cannot be used as a marker for peroxisome degradation, for instance when using a mutant in which matrix protein import is strongly impaired (e.g., a *pex* mutant), the fate of peroxisomal membrane proteins should be analyzed.

### 4.1.3. Analysis of the levels of peroxisomal matrix proteins as a marker for peroxisome degradation

In *H. polymorpha* cells grown on methanol as the sole carbon source, AO is an abundant peroxisomal matrix protein, constituting 5%–30% of the total cellular protein depending on growth conditions. The relatively high abundance of AO renders it an excellent marker to study the fate of peroxisomes after induction of peroxisome degradation, either by following the reduction of the level of AO protein over time (by Western blotting) or by determining the decrease in AO-specific enzyme activities.
(described subsequently). For analysis by Western Blotting, successive samples of a time series of cells that were transferred to pexophagy-inducing conditions are loaded on 10% SDS-PAA gels. After Western Blotting by standard protocols, a specific α-AO primary rabbit antiserum is used, followed by an alkaline phosphatase-conjugated secondary antirabbit antibody using NBT-BCIP as substrate for the detection of the immunogenic protein bands. In the protein samples taken after induction of pexophagy, the intensity of the AO band (running at \( \pm 70 \) kDa) should decrease with time, which can be quantified by densitometry scanning of the blots. Additionally, degradation of AO results in the appearance of lower molecular weight degradation bands on the blot (Fig. 14.2). Because AO is inactivated during peroxisome degradation (Bruinenberg et al., 1982), generally the kinetics of the decrease in enzyme activities is faster relative to the decrease of AO protein (see the subsequent section).

4.1.4. Analysis of the levels of peroxisomal membrane proteins as a marker for peroxisome degradation

Next to monitoring the fate of a peroxisomal matrix protein by Western Blotting, the decrease in the level of peroxisomal membrane proteins can also be used. However, proteins containing domains exposed to the cytosol might also be susceptible to other modes of degradation (e.g., via the ubiquitin–proteasome pathway). Before using a novel membrane protein as a tool to study pexophagy, its turnover by alternative pathways should be determined (e.g., by studying the change in levels upon induction of peroxisome degradation in an \( \text{atg}1 \) mutant).

\( H. \text{polymorpha} \) Pex10 is a suitable peroxisomal membrane marker for degradation studies (Veenhuis et al., 1996). However, because of the generally low levels of Pex10, the sensitive chemiluminescent horseradish peroxidase method is preferred (Roche BM Chemiluminescent western Blotting Kit) over the alkaline phosphatase–based method.

In addition, a constructed fusion protein consisting of the first 50 amino acids of the peroxisomal membrane protein Pex3 fused to GFP

---

**Figure 14.2** Western blot, prepared of crude extracts of methanol-grown \( H. \text{polymorpha} \) cells exposed for the indicated time points to 0.5% glucose, using an α-AO specific antiserum. AO protein levels decrease in time, while also characteristic AO degradation products (arrowheads) are observed.
N50.Pex3.GFP, expressed under control of the inducible AOX promoter, has been successfully used as a marker (van Zutphen et al., 2008; see Fig. 14.3). The GFP portion of the fusion protein forms a rather stable barrel structure, which is degraded relatively slowly in the vacuole. The formation of cleaved N50.Pex3.GFP can also easily be monitored by Western blotting or fluorescence methods (Shintani and Klionsky, 2004).

4.1.5. Nonperoxisomal control proteins
To determine the specificity of the peroxisome degradation process, turnover of other components in the cell should also be analyzed. This has been performed mainly by Western blot analysis. As markers for mitochondria, porin or malate dehydrogenase may be used, whereas as a representative of the endoplasmic reticulum, the levels of Sec63 may be analyzed (Kiel et al., 1999). As marker for the cytosol translation elongation factor 1-α (eEF1A) and Hsp70 may be applied successfully (Bellu et al., 2001b).

4.1.6. Preparation of cell-free extracts for specific enzyme activity measurements
Specific enzyme activity measurements to follow peroxisome degradation are performed in crude cell free extracts, which are prepared as follows.
Protocol

1. Harvest a culture of cells, grown under the appropriate conditions as described previously to induce pexophagy, with a volume corresponding to approximately 10 OD\textsubscript{660} units (volume × OD\textsubscript{660}) and cool on ice.
2. Centrifuge the cells for 5 min at 3000 g and wash the pellet twice (by resuspending and centrifugation) using 10 ml of 50 mM potassium phosphate buffer, pH 7.2.
3. Resuspend the cells in 500 µl of potassium phosphate buffer and lyse the cells using a vortex or Fastprep (FP120, Bio101/Savant, Qbiogene, Cedex, France) after adding 0.5 volume of acid-washed glass beads for 1 min (see chapter 1), followed by cooling on ice for 1 min. This is repeated until the majority of the cells are broken (checked by light microscopy).
4. Remove unbroken cells and cell debris by centrifugation for 5 min at 14,000 rpm at 4 °C. The resulting supernatant can be used for enzyme activity measurements.

4.1.7. Alcohol oxidase activity assay

Alcohol oxidase catalyzes the oxidation of methanol, thereby producing formaldehyde and hydrogen peroxide. The hydrogen peroxide that is generated can be assayed via the oxidation of reduced ABTS (2,2\textprime;-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) depicted in formula 1. This reaction is catalyzed by peroxidase. The end product, oxidized ABTS, has a green color with an absorption maximum at 420 nm (Verduyn et al., 1984).

\[
\begin{align*}
\text{CH}_3\text{OH} + \text{O}_2 & \rightarrow \text{H}_2\text{CO} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 2 \text{ABTS}_{\text{red}} & \rightarrow \text{H}_2\text{O} + 2 \text{ABTS}_{\text{ox}}
\end{align*}
\]

Protocol

1. Add an appropriate amount of buffer A (0.5 mg/ml ABTS, 10 U/ml horseradish peroxidase, in 50 mM potassium phosphate buffer, pH 7.2) to a glass cuvette such that together with the sample the final volume will be 990 µl. Place the cuvette into the thermostated cuvette holder of a spectrophotometer (temperature set to 37 °C).
2. Add the required amount of cell free extract (as prepared in section 4.1.6).
3. Record absorbance at 420 nm (A\textsubscript{420nm}) and equilibrate until the absorbance remains constant.
4. Add 10 µl of 10 M methanol (40% v/v) and mix.
5. Measure the change in absorbance at 420 nm.
6. Perform the assay in triplicate using 3 different sample volumes. All three measurements should result in similar specific activities.
7. Calculate the enzyme activity using the following equation:

\[
\text{Units/mg} = \frac{V}{2.\varepsilon.d.v.c} \times \frac{\Delta t}{\Delta E}
\]

where

\[
\begin{align*}
V &= \text{total volume (ml)}, \\
v &= \text{sample volume (ml)}, \\
\varepsilon &= \text{extinction coefficient (cm}^2/\mu\text{mol}) = 43.2 \text{ cm}^2/\mu\text{mol}, \\
d &= \text{length of the light path}, \\
c &= \text{protein concentration of the sample in mg/ml}, \\
\Delta E &= \text{change in absorbance}, \text{ and} \\
\Delta t &= \text{change in time (min)}.
\end{align*}
\]

4.2. Morphological analysis

Uptake of peroxisomes by the vacuole during autophagy can be visualized by electron microscopy or, after introduction of a fluorescent peroxisomal marker, by fluorescence microscopy.

4.2.1. Fluorescence microscopy

To visualize peroxisomes, fluorescent proteins are used that are either sorted to the peroxisomal lumen or to its surrounding membrane. The use of a fluorescent matrix protein is preferred as it is not susceptible to degradation by processes other than autophagy. The most common peroxisomal matrix targeting signal is the C-terminal PTS1 (–SKL). Thus, green fluorescent protein (GFP) fused to SKL localizes to the peroxisomal matrix in \textit{H. polymorpha}. During peroxisome degradation, the matrix contents are released into the vacuolar lumen, as observed in Fig. 14.4.

A suitable peroxisomal membrane marker is the fusion protein N50. Pex3.GFP (see section 4.1.4). This marker can also be used to tag peroxisomal membranes in mutants defective in matrix protein import (see Fig. 14.4; for details, see van der Klei and Veenhuis 2007).

To demonstrate that peroxisomes have indeed been taken up by the vacuole, the vacuolar membrane can be specifically stained with the red fluorescent dye FM 4-64 (N-(3-triethylammoniumpropyl)-4-(6-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide, Molecular Probes Invitrogen). To label with FM 4-64, 0.5 to 1 ml of culture is incubated with 1 μl of FM 4-64 solution (100 μg dissolved in 83 μl DMSO, 1 μg/μl) for at least 45 min at 37 °C with shaking at 200 rpm, followed by washing with prewarmed media (also see chapter 7). Because FM 4-64 is taken up by endocytosis, the relatively slower-growing methanol cells need to be incubated longer than cells cultivated on glucose.

4.2.2. Ultrastructural analysis of pexophagy by electron microscopy

Electron microscopy allows for the obtaining of detailed morphological information on peroxisome degradation. Two types of fixation techniques are routinely used prior to embedding the cells for electron microscopy.
Of these, potassium permanganate fixation is suited to visualize overall cell morphology, in particular membranes (Fig. 14.5A), whereas aldehyde fixations are generally used for immunocytochemistry (Fig. 14.5B).

**Protocols**

**KMnO₄ fixation**

All incubations are performed at room temperature unless otherwise indicated.

1. Grow cells under pexophagy-inducing conditions and harvest a volume of the culture corresponding to 10–20 OD₆₆₀ units.
2. Wash the cells three times with 5 ml of demineralized water by resuspending/centrifugation (5000×g 2 min, 10-ml tubes) and subsequently
resuspend the pellet in 5 ml of KMnO₄ solution (1.5% KMnO₄ in water).

3. Incubate the cell suspension for 20 min at room temperature and shake gently every 5 min.

4. After incubation, collect the cells by centrifugation and wash with demineralized water until the supernatant is colorless (3 times with 5 ml of water each usually suffices).

5. Resuspend the pellet in 5 ml of uranyl acetate solution (0.5% in water) and centrifuge for 15 min at 5000×g to obtain a firm pellet. The supernatant should not be discarded but left on top of the pellet for at least 4 h or maximally overnight at room temperature.

6. Decant the uranyl acetate supernatant from the pellet and dehydrate the cells by incubating the pellet with solutions of increasing ethanol concentrations according to the following plan:
   - 15 min in 50% ethanol (without resuspending, the pellet remains intact).
   - 15 min in 70% ethanol (the pellet is broken into small pieces, approximately 1–5 mm³, using a spatula. Incubation is performed without rotation; therefore, the pieces will be lying at the bottom of the tube and the solution can be poured of directly after incubation).
   - 15 min in 96% ethanol (mix carefully, do not use a vortex, and the small pieces should stay intact).

Figure 14.5 (A) Ultrathin section of a KMnO₄-fixed H. polymorpha cell, 30 min after glucose-induced macropexophagy showing sequestration of the large peroxisome (AP) in the cell leaving the smaller organelle (P) unaffected. (B) Ultrathin section of a glutaraldehyde-fixed H. polymorpha cell, labeled with an α-AO antiserum, showing labeling of a peroxisome (P) as well as autophagic vacuoles (V). Key: AP, autophagosome; P, peroxisome; N, nucleus; M, mitochondrion; V, vacuole. The bar represents 0.5 μm.
• 15 min in 100% ethanol (mix carefully).
• Refresh the 100% ethanol solution and incubate for another 30 min.

7. In the subsequent steps, the cells are impregnated with Epon resin. To prepare the Epon embedding resin, mix 100 g of Epon 812 (glycid ether) with 92 g of methylnadic anhydride (MNA), then add 2.3 g of 2,4,6-tri (dimethylaminomethyl) phenol (DMP-30). During the incubations with Epon/ethanol mixtures or pure Epon solutions the tubes are continuously mixed using a slowly rotating incubator. Incubate the samples (i.e., pieces of cell material) with approximately 5 ml of each of the Epon/ethanol mixtures according to the following:
• 4–8 h in a 1:1 mixture of 100% ethanol and Epon
• Overnight in a 1:3 mixture of 100% ethanol and Epon
• 1 h in pure Epon solution
• Refresh the Epon solution and incubate for another 8 h

8. Fill gelatin capsules {3/4} full with pure Epon and load one piece of sample onto the top of the capsule; it will readily sink.

9. Polymerize the Epon by incubating the capsules for 24 h at 80 °C.

10. Prepare sections using a diamond knife and view the sections in a transmission electron microscope.

**Aldehyde fixations**

All steps are performed at 4 °C.

1. Harvest at least 20 OD<sub>660</sub> units of a fresh culture by centrifugation (3 min at 5000×g).

2. Wash the cells 3 times with demineralized water and add 5 ml of either one of the fixation solutions:
   3% glutaraldehyde in 0.1 M Na-cacodylate, pH 7.2
   3% formaldehyde in 0.1 M Na-cacodylate, pH 7.2
   0.5% glutaraldehyde + 2.5% formaldehyde in 0.1 M Na-cacodylate, pH 7.2

   3% glutaraldehyde is the preferred fixative. However, glutaraldehyde may affect the antigenicity of specific proteins. In that case formaldehyde or a mixture of formaldehyde and glutaraldehyde can be used.

3. Carefully resuspend the cells (by manual shaking) in the fixative solution and incubate for 2 h on ice. Mix the suspension every 15 min by inversion.

4. Collect the cells by centrifugation (see previous section) and discard the supernatant fraction. Wash the pellet using fresh Na-cacodylate buffer keeping the pellet intact.

   All subsequent steps are performed at room temperature.

5. Wash the intact pellet of fixed yeast cells twice with demineralized water.
6. Add 5 ml of a freshly prepared solution of 0.4% (w/v) Na-periodate in water to the pellet (keep pellet intact).
7. After incubation for 15 min on a slowly rotating incubator, wash the pellet twice with 5 ml of demineralized water.
8. Incubate the pellet in 5 ml of a 1% (w/v) NH₄Cl solution in water for 15 min at room temperature.
9. Decant the NH₄Cl solution and wash the pellet once with demineralized water.
10. Dehydrate the cell material as indicated above for permanganate fixation. The samples are now ready for impregnation with Unicryl, the preferred plastic for immunocytochemical purposes.
11. Incubate the pieces successively as indicated in the following:
   - 3 h in 5 ml of a 1:1 solution of 100% ethanol and Unicryl
   - 1 h in 5 ml of pure Unicryl
   - Overnight in 5 ml of pure Unicryl
   - 6–8 h in 5 ml of pure Unicryl
12. Embed the material in BEEM capsules (Standard polyethylene embedding capsules size 00; 1×1-mm flat bottom) filled ¾ full with Unicryl. Only use carefully dried capsules! (Dry overnight in 37 °C oven).
13. Polymerize the Unicryl for 2 days using ultraviolet light at 4 °C.
14. Fill the capsules completely with Unicryl and incubate for 2 more days at 30 °C.
15. Cut sections using a diamond knife and transfer sections onto nickel grids (400 mesh, Formvar/carbon coated).

Immunocytochemical staining methods are performed on ultrathin sections that are collected on Formvar/carbon coated nickel grids (do not use copper grids). The incubation steps are performed by floating the grids, section side down, on top of small droplets of the solution on a sheet of Parafilm. All steps are performed at room temperature unless stated otherwise.
16. Incubate the grids with the following solutions: 0.5% BSA in PBS-glycine buffer for 5 min as a blocking step (PBS/Glycine/BSA buffer = 2g/l sodium chloride, 0.05g/l potassium chloride, 0.36g/l disodium hydrogen phosphate, 0.055g/l sodium dihydrogen phosphate, 0.375g/l glycine, 0.025g/l sodium azide and 5g/l BSA).
   Transfer grids to a droplet of appropriately diluted primary antibody in PBS-glycine buffer containing 0.5% BSA and incubate for 1 h at room temperature (alternatively this step can be performed overnight at 4 °C). The appropriate dilution of the primary antibody is generally 10 times less than that used for Western blotting (e.g., 1:100 if a 1:1000 dilution is the optimal dilution for Western blotting).
17. Rinse the grids with PBS-glycine buffer, 3 times for 5 min each.
18. Incubate the grids with a solution of secondary antibodies conjugated to gold in PBS-glycine buffer containing 0.5% BSA (use dilution as
recommended by the manufacturer). Use the appropriate secondary antibodies (i.e., goat-antirabbit-(GAR)-gold when the primary antibodies were raised in goat or goat-antimouse-gold, when the primary antibodies were raised in mice).

19. Rinse the grids in PBS-glycine buffer, 6 times for 5 min each.
20. Rinse grids in distilled water, 4 times for 5 min each.
21. Remove excess water by carefully tipping one side of the grid (section side up) onto filter paper.
22. Poststain the sections by placing the grid (section side down) onto a droplet of 1% uranyl acetate and 0.2% methylcellulose for 20 s.
23. Remove excess staining solution using filter paper and allow the grid to dry.

5. Concluding Remarks

The success of electron microscopy (EM) studies, much more than biochemical approaches, depends on the availability of fast-growing cells that preferably are in the exponential growth phase. To avoid synthesis of storage products (e.g., glycogen) that strongly interfere with optimal cell architecture, we aim to avoid culturing cells at high concentrations of carbon sources (always <1% glucose). Similar arguments hold for fluorescence microscopy studies aiming at live cell imaging. That is, the cellular processes are optimally visualized when the cells have not been exposed to high concentrations of the carbon source.

It should also be noted that the resolution of fluorescence techniques is still far from the resolution of EM techniques. This may in specific cases lead to overinterpretation of the data. It is therefore recommended that, wherever possible, the two methods are used in parallel.

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