Investigation of the ultrastructure of yeast using cryo-immuno-electron microscopy

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Yeast has been a crucial model system for the investigation of a multitude of cellular processes and many approaches have been used to study these, including genetics, biochemistry and fluorescence microscopy approaches. The morphological analyses of these organisms by electron microscopy (EM), however, have remained limited because of the lack of techniques leading to satisfactory ultrastructural resolution. In this compendium, we will review the various applications of an immuno-electron microscopy (IEM) protocol we have recently developed from the Tokuyasu method. This novel approach, which allows excellent cell preservation and unprecedented resolution of yeast morphology, has been successfully applied not only for the study of *Saccharomyces cerevisiae* and *Pichia pastoris* but also the filamentous fungus *Aspergillus nidulans*. This procedure is compatible with immuno-gold labelling and allows the combination of protein localization with a fine ultrastructural resolution of protein complexes, vesicular carriers and organelles. This new IEM protocol is thus a valuable tool for the large community of scientists studying the physiology of yeast but also exploiting these organisms as model systems for the investigation of molecular principles important cellular pathways conserved among eukaryotes.

**Keywords** *Saccharomyces cerevisiae*, *Pichia pastoris*, *Aspergillus nidulans*, electron microscopy, Tokuyasu cryosectioning, immuno-gold labelling, rehydration method, high-pressure freezing, positively charged Nanogold

1. Introduction

*Saccharomyces cerevisiae* but also other yeast species are crucial model systems for the study of a multitude of cellular processes due to their simple and rapid growth, and their amenability to genetics, molecular biology and biochemical procedures. Despite the variety of possible experimental approaches, including fluorescence microscopy (FM), the routine incorporation of the morphological analyses has remained difficult. EM has proven to be a powerful tool in the exploration of high eukaryotes at the ultrastructural level extending the possibilities of FM. In particular, the resolution of FM, which is in the order of 500 nm, even when optimized by computer assistance, is far too low to resolve the morphology of organelles such as the Golgi, the trans-Golgi network, endosomes and transport vesicles. In addition, reallocation of proteins through loss of cellular integrity during sample preparation is a potential flaw in FM. By contrast, EM combined with immuno-gold labeling has the capacity to simultaneously show the precise location of a protein and its ultrastructural environment at nanometer resolution.

2. Exploration of the yeast ultrastructure using EM

2.1. The different EM approaches that have been applied to yeast [1-3]

The choice of EM techniques applied to yeast depends on the specimen and the analysis required (Fig.1). The first step of the procedure involves the cell immobilization to keep all constituents of cells as close as possible to the natural state. Fixation can be achieved by chemical bounds or rapid freezing. The most commonly used method to fix biological specimens is the chemical fixation achieved by chemical crosslinking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide. Nevertheless, aldehyde fixation may display some inconvenient effects. It is relatively slow since fixatives have to diffuse into the cell and in addition, these chemicals could change the local pH, disturb the ionic balances and affect the osmolarity in the different organelles [4, 5]. To bypass these problems, a good alternative is the cryo-immobilization (or physical fixation) through High Pressure Freezing (HPF). This allows the rapid freezing in milliseconds of the sample in liquid nitrogen under very high pressure to avoid ice crystal formation (Fig.1).

After fixation, cells have to be embedded into a rigid support. This step is necessary because high resolution imaging requires thin layers of cells which can only be cut from very hard blocks. Cells can be embedded in a resin for conventional EM or in gelatin when processed for IEM on ultrathin cryosections. The overall ultrastructure of yeast has been often approached with methodologies that lead to the embedding of the specimen in a resin (Fig.1; [6-9]). Fixation and subsequent embedding in resins such as Spurr’s and Epon, has allowed for obtaining satisfactory results in preserving the yeast morphology. With conventional EM approaches, the more prominent subcellular compartments such as the nucleus or the vacuoles are readily detectable while small organelles, including the different Golgi compartments or the various classes of endosomes, cannot be easily seen. Nevertheless, conventional EM can be very
useful to monitor apparent morphological changes such as ER proliferation the, Golgi apparatus expansions, endosome enlargements or autophagosome formation [10-14]. Conventional EM can also be combined with immuno-gold labelling when using particular embedding resins such as LR white or Lowicryl K4M/HM20 (Fig.1; [1, 8, 15, 16]), though they do not allow satisfactory membrane resolution and contrast. In addition, only a limited number of proteins can be labelled with immunological reactions as fixation conditions, dehydration procedures, resin properties and the resin polymerization temperature have cumulative denaturing effects on epitopes. HPF followed by resins results in a better membrane resolution but it does not optimally preserve the epitopes necessary for immunological reactions [6, 17].

To circumvent these problems and obtain high-resolution, the best established and generally the most efficient method for localization of proteins by immuno-gold labelling in mammalian cells and tissues, is the Tokuyasu cryosectioning, a technique based on chemical fixation with aldehydes and named after its originator (Fig.1; [18, 19]).

Figure 1. EM procedures for the exploration of yeast ultrastructure. Schematic representation of the main EM and IEM approaches used to explore the morphology and protein localisation at the ultrastructural level in yeast.
2.2. The challenges of yeast

One of the major challenges in the processing of yeast for EM investigations has been the relative impermeability of the cell to both fixatives and resins due to the presence of a cell wall. An additional issue linked to the cell wall is its detachment and/or flipping over the section during the cryosectioning, which damages the specimen. This phenomenon is probably caused by tension release during the sectioning due to the fact that the yeast cell wall is a rigid extracellular layered meshwork formed of glucans, chitin and mannoproteins.

Another major obstacle for yeast ultrastructural analyses is the high protein concentrations in the cytoplasm. Most EM contrast methods exploit the fact that protein concentration in the membranes is higher than that of the cytoplasm. This difference enhances the contrast leading to the resolution of the various intracellular compartments. In yeast, however, this difference is limited, leading to less defined membrane outlines.

2.3. EM adaptations to yeast

To bypass the problems of bad infiltration with viscous EM chemicals and cell wall rigidity, two different approaches have been used in the past to embed yeast cells. The first approach consists of the digestion of the cell wall by action of enzymatic enzymes before fixation [20]. The second approach is a post-fixation treatment of the cells with metaperiodate which oxidizes the glucan matrix composing the cell wall and renders it more permeable to viscous compounds [16]. Importantly, these two methods also provide a solution to the problem of the cell wall detachment and its flipping over the section during the sectioning.

The problem of contrast due to the high protein concentration of the yeast has been solved by adapting the Tokuyasu cryosectioning method to yeast [7]. The “negative staining” resulting from the lipid extraction leads to an outstanding definition of membranes (see below).

3. A novel procedure of IEM on thin cryosections

In 2008, our laboratory has developed a new IEM procedure adapted from the Tokuyasu method to prepare cryosections from mildly fixed cells [7]. In particular, we introduced a post-fixation step where cells are treated with metaperiodate in order to permeabilize the cell wall. The metaperiodate treatment allows better infiltration of the cryoprotectants and reduces specimen damage caused by a rigid cell wall. As a result, the optimal cell preservation, combined with the staining of the membranes typical of the Tokuyasu method, create a superb contrast that leads to a unique resolution of the yeast morphology [7]. Because epitopes are also well preserved [18, 19], our IEM protocol permits a combination of efficient localization studies with optimal structural resolution, vesicular carriers and organelles (Fig.2).
Figure 2. IEM analysis on *Saccharomyces cerevisiae*. Wild type cells expressing Atg9-GFP (A and B), Vrg4-3xHA (C) or Idh1-3xHA (D) were grown to logarithmic phase and processed for IEM following the adapted Tokuyasu method adapted to yeast [2]. Cryosections were immuno-gold labelled with anti-CPY (A), anti-GFP (B) and anti-HA (C and D) antibodies. A. The specifically vacuole labelled for CPY is delimiting by a white membrane and has a characteristic dark grey content. B. Atg9 concentrates to a cluster of vesicles and tubules often found adjacent to mitochondria [21]. C. The *S. cerevisiae* Golgi appears as a tubular monocisterna with a horseshoe shape. D. Mitochondria are readily identifiable at the ultrastructural level and protein marker Idh1 can specifically be used to immuno-gold labelled them. The size of the gold particles is indicated on the top of each picture. CW, cell wall; G, Golgi; M, mitochondria; N, Nucleus; PM, plasma membrane; V, vacuole. Bar, 200 nm.

3.1. The protocol

The general procedure to prepare samples for the Tokuyasu method can be divided into four main steps: 1. Fixation, 2. Embedding into gelatine, 3. Protection by sucrose infiltration and 4. Freezing in liquid nitrogen [19]. The strategy to develop a protocol suitable for the analysis of yeast and bypassing the problems inherent to yeast, has been to improve and optimize the routinely used protocol for mammalian cells [7]. Briefly, in this new protocol cells are first fixed with a mixture of formaldehyde/glutaraldehyde for 3h. This initial step is followed by the treatment of the fixed cells with 1% periodic acid for 1h to permeabilize the cell wall. All solutions are made in PHEM buffer [20 mM piperazine-1,4-bis(2-ethanesulfonic acid)/PIPES, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/HEPES, pH 6.9, 20 mM ethylene glycol tetra-acetic acid/EGTA, 4 mM MgCl₂], which helps to preserve the immuno-reactivity of the yeast preparation and does not interfere with the membrane contrast. Subsequently, the samples are embedded and solidified in 12% gelatin before being infiltrated with 2.3 M sucrose. This is one of the most critical steps of the Tokuyasu method because the cryo-protectant properties of this sugar permit the freezing of biological material without the formation of morphology damaging crystals. The last step of the procedure is the freezing of the samples by immersion in liquid nitrogen, where samples can be kept for several years.

Ultrathin cryosections are then cut with cryo-ultramicrotomes at -120°C and placed on formvar/carbon-coated grids. The cryosections can then be either directly imaged after coloration or immuno-gold labelled to combine ultrastructural analysis and protein localization [18]. For the immuno-gold labeling, the sections on grids are first incubated with the primary antibody and subsequently with gold particles conjugated to either secondary antibodies or protein A.
membrane contrast is performed using a uranyl solution at pH 7 for 5 min followed by 5 min incubation in a mixture of methylcellulose-uranyl [22].

3.2. Documented applications
This new IEM techniques has been successfully used in different studies. Using this procedure and Atg9 as a protein marker of at least part of the membranes used to form autophagosomes, we have been able to acquire insights into the crucial question of the membrane origin and organization of the yeast phagophore assembly site, the precursor structure of autophagosomes [21]. In addition, the same IEM approach has suggested that the Golgi could also supply part of the autophagosomal membranes because a block of the sorting functions of this organelle leads to an accumulation of phagophores, one of the intermediates of autophagosome biogenesis [23]. This protocol has also been used to study the involvement of a novel nucleoporin in the nuclear pore organisation [24]. Finally, certain functions of the yeast endosomal system have been explored using this IEM procedure combining the ultrastructural analysis with the localization of both endosomal cargo and resident proteins [25, 26, 27].

4. Adaptations of the IEM protocol for other applications

4.1. HPF and the rehydration method
The disadvantage of HPF is that subsequent to the freezing, samples undergo a long fixation time, dehydration and resin embedding. Compared to sections embedded in gelatin obtained with the Tokuyasu method, visualization of yeast membranes in resin sections is relatively poor and the immuno-labeling efficiency is generally much lower or destroyed [7]. Recently, a novel IEM procedure called the rehydration method, has been developed [28]. This approach combines the advantages of HPF and those of the Tokuyasu technique, e.g. rapid sample immobilization and excellent membrane resolution/immuno-labelling. We have successfully integrated the rehydration method with our IEM protocol and the results, e.g. the quality of the ultrastructural resolution of the yeast morphology and the efficiency of the immuno-labeling specificity, are also excellent [7]. This approach could be valuable for the analysis of structures such as vacuoles that are not perfectly immobilized and/or preserved by chemical fixation.

4.2 Labelling of the endosomal system with positively-charged Nanogold particles
Although the yeast endosomal system has been extensively studied in yeast using biochemical and FM methods, investigations at the ultrastructural level have been minimal for two mains reasons: first, the non-optimal resolution of yeast EM preparations and second, the absence of protein markers that can be detected by EM [29]. A successful approach employed to visualize the different compartments of the endosomal system has been the internalization of positively-charged Nanogold particles by yeast spheroplasts [29]. These particles strongly bind the negatively charged lipids present on the cell surface and after endocytosis, the Nanogold reaches the vacuole after passing through the various endosomal organelles (e.g. early endosomes, late endosomes and multivesicular bodies). After uptake, the Nanogold particles are visualized by performing a silver enhancement reaction on the sections [29]. By integrating the Nanogold labelling with our IEM protocol, we have generated a procedure that produces excellent results when applied for the examination of early and late endosomes, and of mutants with an endosomal trafficking defect [30]. Importantly, this method is compatible with immuno-gold labeling of protein markers, and it is consequently appropriate for localization studies of both resident and cargo proteins [30]. This novel protocol has already been applied to study the role of the various subunits of the CORVET (class C core_vacuole/endosome tethering) complex in the tethering and organization of yeast late endosomal membranes [25].

5. Application for the study of other yeast and fungi
The fact that our protocol permits high quality cryosections from organisms surrounded by a cell wall makes it suitable for the analysis of other yeast than S. cerevisiae as well as several other types of fungi. We have thus far successfully used it for investigations in both: P. pastoris, a methylotrophic yeast mostly used to study the biogenesis and turnover of peroxisomes, and in the filamentous fungus A. nidulans, which has played a central role in the exploration of eukaryote genetics [31-35]. Although our IEM protocol did not require any changes when applied to P. pastoris, the hyphal growth by apical extension of A. nidulans required few adaptations (in preparation). Conidiophores of this fungus are inoculated in the center of solid medium-coated glass slides, which initiates the filamentous growth and distribution of A. nidulans [36]. Subsequently, the glass slides are gently immersed in a bath of fixative before being treated with periodic acid. Cell embedding also occurs on the slides and after having cut small blocks of agar, samples are processed following the standard protocol for IEM on thin cryosection. The ultrastructural resolution of P. pastoris and A. nidulans cells are highly satisfactory and allow a clear identification and analysis of several subcellular
organelles (Fig.3). Importantly, both \textit{P. pastoris} and \textit{A. nidulans} preparations can efficiently be immuno-gold labeled as well.

\begin{figure}[h]
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\caption{Application of the IEM procedure for yeast to \textit{P. pastoris} and \textit{A. nidulans}. \textit{P. pastoris} (A and B) and \textit{A. nidulans} (C and D) were grown in rich medium and processed for IEM following adaptations of Tokuyasu method. A,B. Micrograph displaying the overall morphology of \textit{P. pastoris}. C,D. Ultrastructural details of \textit{A. nidulans}. CW, cell wall; ER, endoplasmic reticulum; G, Golgi; M, mitochondria; N, Nucleus; P, peroxisomes; PM, plasma membrane; V, vacuole; WB, Woronin body; S, septum. Bar, 200 nm.}
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6. Conclusions and future directions

EM has been an essential technique to acquire knowledge about intracellular protein trafficking and the cells ultrastructural organization in high eukaryotes. This approach is particularly important in yeast as well when investigating the compartments of the endosomal and secretory systems because in FM they appear as puncta. This experimental need, has been hindered by the lack of however procedures allowing satisfactory results. Our protocol of IEM on thin cell cryosection fills this gap because it generates samples with an excellent membrane resolution that can be immuno-gold labelled as well.

An emerging field in EM is the use of the electron tomography to study the three-dimensional projection of a structure or an organelle [37, 38]. Although this technique has already been applied to yeast [39], it has not yet been combined with immunological reactions as done for mammalian cells [40]. Consequently, it will be very useful to combine our cryo-immuno-gold labelling method with electron tomography to recognize specific structures or compartment subdomains, and reconstruct their membranous surrounding.

In conclusion, our IEM protocol, which exploits the high resolution and the efficient immuno-gold labelling of the Tokuyasu method, can be applied in the laboratory of fungi with excellent results. That, added to the possibility of combining this procedure with others EM techniques such as HPF or Nanogold labelling, provides the scientific community with a new and flexible experimental tool for the investigation of numerous low eukaryote model organisms.

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References


[15] Murray S. High pressure freezing and freeze substitution of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* for TEM. *Methods Cell Biol.* 2008;88:3-17.


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