Video Article

Nanogold Labeling of the Yeast Endosomal System for Ultrastructural Analyses

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URL: http://www.jove.com/video/51752
DOI: doi:10.3791/51752

Keywords: Cellular Biology, Issue 89, positively charged nanogold, silver enhancement, Tokuyasu procedure, electron microscopy, immunogold labeling, yeast

Date Published: 7/14/2014

Citation: Mari, M., Griffith, J., Reggiori, F. Nanogold Labeling of the Yeast Endosomal System for Ultrastructural Analyses. *J. Vis. Exp.* (89), e51752, doi:10.3791/51752 (2014).

Abstract

Endosomes are one of the major membrane sorting checkpoints in eukaryotic cells and they regulate recycling or destruction of proteins mostly from the plasma membrane and the Golgi. As a result the endosomal system plays a central role in maintaining cell homeostasis, and mutations in genes belonging to this network of organelles interconnected by vesicular transport, cause severe pathologies including cancer and neurobiological disorders. It is therefore of prime relevance to understand the mechanisms underlying the biogenesis and organization of the endosomal system. The yeast *Saccharomyces cerevisiae* has been pivotal in this task. To specifically label and analyze at the ultrastructural level the endosomal system of this model organism, we present here a detailed protocol for the positively charged nanogold uptake by spheroplasts followed by the visualization of these particles through a silver enhancement reaction. This method is also a valuable tool for the morphological examination of mutants with defects in endosomal trafficking. Moreover, it is not only applicable for ultrastructural examinations but it can also be combined with immunogold labelings for protein localization investigations.

Video Link

The video component of this article can be found at http://www.jove.com/video/51752/

Introduction

The endosomal system is a major membrane sorting apparatus that plays multiple crucial cellular roles including trafficking of lysosomal enzyme sorting receptors and the recycling of plasma membrane (PM) receptors[1,2]. Endosomes are divided in three different compartments, *i.e.* the early endosomes (EE), the late endosomes (LE) and the recycling endosomes. This classification is based on the time it takes for endocytosed material to reach them, on specific marker proteins and on their morphology. Membranes, *i.e.* protein and lipid bilayers, internalized from the PM can either be delivered to lysosomes via endosomes for degradation or be recycled back. Membranes are also transported to endosomes from the Golgi and similarly, either continue to lysosomes or be retrieved back to the Golgi. Furthermore, proteins can be sorted into luminal vesicles budding inward from the endosomal limiting membrane, a process that leads to the formation of a subcategory of LE, the multivesicular bodies.

The yeast endosomal system is relatively less complex than the one of high eukaryotic cells. Yeast endosomes are divided into EE and LE. In contrast to mammalian cells they do not contain recycling endosomes but also tissue-specific lysosome-related organelles. Consequently they have a less complex network of endosomal trafficking routes[3,4]. Therefore yeast has represented and still represents an advantageous experimental system to study some of the principles underlying membrane traffic in the endosomal system. This advantage is emphasized by the fact that numerous genes involved in the endosomal pathways have been initially isolated with genetic screens in yeast[5]. While the yeast endosomal system in wild type and mutant cells has been extensively studied using biochemical and fluorescence microscopy approaches, its investigation at the ultrastructural level has only been minimal. Morphological analyses are particularly relevant in yeast because most of the endosomal organelles are detected as punctuate structures by fluorescence microscopy, which make difficult their unequivocal identification[6]. Unfortunately only a limited number of antisera recognizing yeast endosomal protein markers is working in immuno-electron-microscopy (IEM) preparations[7-10]. For some proteins, this problem has been circumvented by the endogenous tagging of the gene of interest and the use of an antibody recognizing the tag to detect it[7,11,12]. Often, however, proteins are undetectable by IEM because of their low expression levels. Their overexpression is not a solution because this approach can induce mis-localizations and/or alterations in the organelle morphology/functions. Thus the labelling of the endocytic compartments with a probe detectable by electron microscopy EM is an effective option. This is an optimal solution especially if the probe is entering the endocytic route in a time-dependent manner, which allows to know when it will mark a specific organelle[6].

The uptake of positively charged nanogold by yeast spheroplasts (*i.e.* yeast where the cell wall has been enzymatically removed) has successfully been used to identify the yeast endosomal compartments[10]. These particles strongly bind to the negatively charged lipids composing the biological membranes. Thus the positively charged nanogold associates with the PM, penetrates the cell by endocytosis and passes through the EE and LE before reaching the vacuole. These small gold particles, however, do not have an appropriate size to be seen by EM. To render them visible, their size can be enlarged by chemical reactions that lead to the deposition of silver or gold around the gold

probe[13-15]. We have developed and successfully applied an IEM approach based on the Tokuyasu method to perform subcellular localization studies[8,16]. This method allows performing immunogold labelling on yeast preparations with an excellent resolution of the morphology[8,17-24]. We have also established a procedure combining this IEM protocol with the nanogold labelling of the yeast endosomal system compartments[6]. Using this approach we have morphologically characterized different subclasses of endosomes and ultrastructurally examined mutants with an endosomal trafficking defect[6,25]. Moreover, we have demonstrated that this nanogold labelling can be combined with immunogold labelings providing the possibility to explore the distribution of a protein of interest on the different endosome subpopulations. Here we present how the labelling of the yeast endosomal system with positively charged nanogold is practically performed.

Protocol

1. Spheroplast Preparation

1. Incubate the yeast overnight at 30 °C in 10 ml of the appropriate medium determined by the design of the experiment.
2. The day after, measure the optical density of the culture at 600 nm (OD_{600}) using a photometer, Dilute cells in the same culture medium to an OD_{600} of 0.2-0.4 and grow them to an exponential growth phase unless the design of the experiment requires a different condition. Cells are in the exponential phase when the culture has an OD_{600} of 1-2.
3. Collect 10 OD_{600} equivalents of cells by centrifugation at 3,500 x g for 5 min in a 50 ml tube. After centrifugation, discard the supernatant.
4. Resuspend the cell pellet in 5 ml of 100 mM PIPES (pH 9.6), 10 mM dithiothreitol and incubated at 30 °C for 10 min.
5. Collect again the cells by centrifugation at 3,500 x g for 5 min. Discard the supernatant.
6. Resuspend the cells in 5 ml of medium (determined by the design of the experiment) containing 1 M sorbitol and 5 mg of lytic enzyme, and incubate the mixture at 30 °C with gentle shaking for 30 min.
7. Centrifuge the cell suspension at 300 x g for 5 min to collect the pellet fraction, which corresponds to the spheroplasts. Discard the supernatant.
8. Resuspend the spheroplasts in 960 µl of ice cold media (medium determined by the design of the experiment) containing 1 M sorbitol and transfer the mixture into an ice cold 2 ml microcentrifuge tube.

2. Nanogold Uptake

1. Using a pipette, gently mix the spheroplast with 4 nmol of positively charged nanogold particles resuspended in 40 µl of water. The final volume of the mixture has to be of 1 ml.
2. Place the obtained cell suspension on ice for 15 min.
3. Transfer the suspension at room temperature and incubate it for the required time to allow nanogold internalization by endocytosis.
   NOTE: A 5 min uptake will principally lead to the labelling of endocytic vesicles and early endosomal compartments, while a 15 min uptake will permit to mark the entire endosomal system (early and late endosomes). Longer incubation times (more than 30 min) will also permit to label the vacuole.
   NOTE: Pulse-chase labelling experiments cannot be performed with this method. Therefore, when labelling for the analysis of LE, nanogold will also be found at the PM and inEE.

3. Fixation and Sectioning

1. Stop the nanogold uptake by adding 1 ml of double strength fixative [4% paraformaldehyde (PFA), 0.4% glutaraldehyde (GA) in 0.1 M PHEM buffer (20 mM PIPES, 50 mM HEPES, pH 6.9, 20 mM EGTA, 4 mM $MgCl_2$)] containing 1 M sorbitol to the cell suspension. Kept the tube at room temperature.
2. Gently invert manually the micocentrifuge tubes several times during 30 min (this will permit to keep the spheroplasts in suspension) and then centrifuge it twice at 1,700 x g for 25 sec.
3. Replace the fixative by discarding the supernatant and by adding 1 ml of fresh standard strength fixative (2% PFA, 0.2% GA in 0.1 M PHEM buffer) containing 1 M sorbitol and incubate for 2 hr at room temperature on a slowly rotating wheel.
4. Process the cells for cryo-sectioning as described previously[6]. NOTE: For the nanogold uptake and silver enhancement procedures, the periodic acid treatment described in the indicated protocol has not to be performed. The periodic acid treatment is to better permeabilize the cell wall[26] but this structure has been eliminated during the generation of spheroplasts.
5. Cut 50 nm thin cryosections at -120 °C with dry diamond knife using an UCT ultramicrotome as previously described[8] and place them on Formvar carbon coated 50 meshes nickel grids.

4. Silver Enhancement for Nanogold Particle Visualization

NOTE: The procedure to prepare the reaction mixtures is basically the one indicated by the manufacturer. Practical adaptations using this protocol, makes the silver enhancement procedure effective and reliable on cryosections.

1. Remove the silver enhancement kit from the freezer and thaw it in a 37 °C incubator or water bath. When thawed, place the kit in a 24 °C incubator placed into a dark room until use (see 4.7).
2. Place the heating plate in the dark room and warm it to the final temperature of 24 °C.
3. Cover the top of heating plate with the surface protector, shiny side up, and secure it with a tape.
4. Place the Parafilm on the Benchkote and mark the edges with a black marker to be able to see the Parafilm edges in the dark.
5. Tape a thermometer on the top of the Benchkote to monitor the temperature of the heating plate. Gradually adjust the temperature if not 24 °C.
6. Place the 50 ml tube with double distilled water and the silver enhancement kit inside.

7. Fill the small Petri dishes with double distilled water, pre-warmed at 37 °C. Place the grids in the water, specimen side down, for 30 min.
8. Repeat this 4.7 step one more time.
9. Transfer the grids in the storage box and bring them to the dark room.
10. Rinse the grids again by passing them (with the specimen side down) on several drops of double distilled water at 24 °C placed on the Parafilm that has been fixed on the heating plate.
11. Make sure that 9 additional double distilled water drops are ready on the Parafilm, for the rinsing after the silver enhancement reaction.
12. Turn the light off and make sure that all lights are off. Turn the red light on.
13. Take out the A and B solutions of the silver enhancement kit from the 24 °C incubator.
14. Put first 6 drops of the A solution and then 6 drops of the B solution in a 1.5 ml microcentrifuge tube, and mix well with a glass Pasteur pipette avoiding to make bubbles. The solution is quite thick and it has to be manually mixed with a pipette before finally vortexing it briefly.
15. Put the A and B solutions back in the 24 °C incubator and take out the C solution.
16. Add 6 drops of the C solution to the A/B mix. Then mix again first with a Pasteur pipette and then by vortexing, avoid making bubbles.
17. Use immediately the final mixture by placing drops (~20 µl/drop) on the Parafilm.
18. With a clean, anti-magnetic tweezers, place the grids on the mixture (*e.g.*, silver enhancing reaction) for 6 to15 min depending of the enhancement (*i.e.* size of the gold particles) wished to be obtained.
19. Remove the grids from the mixture and pass them on the top of the double distilled water drops at 24 °C for washes. First, use in rapidly succession 6 drops and then 3 drops with 7 min incubation time per drop, before turning the lights on.
20. Proceed to the next step, either immuno-gold labeling or membrane staining[27] in order to visualize the result for an EM investigation.

Representative Results

According to the presented protocol, the morphology of the yeast endosome system can be access by transmission EM. **Figure 1** shows different types of endosomal compartments that have been reached and therefore labeled with nanogold. The silver-enhanced nanogold can be clearly seen as electron dense particles. The optimal settings established for the silver enhancement reaction allows having gold particles in a homogenous size range between 5 and 15 nm, which does not interfere with the ultrastructure of the yeast cell. Plasma membrane as well as early endosomal compartments are accessible to the nanogold after a 5 min incubation at 24 °C (**Figure 1A**) while LE, in the presented case multivesicular bodies, are reachable by these particles after at least 30 min of uptake (**Figure 1B**). The resolution power of combining our IEM procedure[8] with the nanogold/silver enhancement protocol described here is underlined by the preservation and quality of the morphology of the shown organelles, in particular the internal vesicles of the multivesicular bodies (**Figure 1B**).

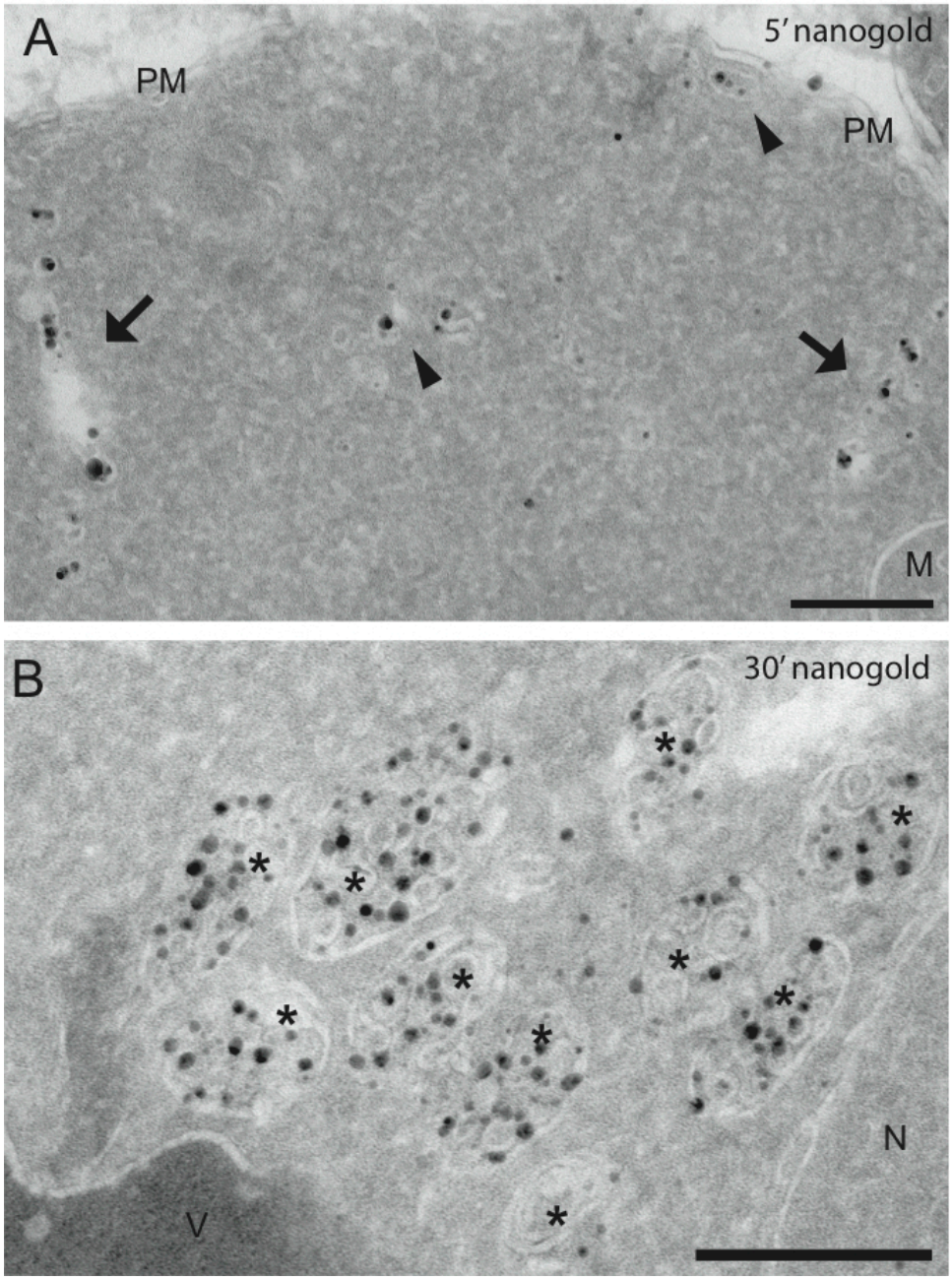

Figure 1. Ultrastructural analysis of nanogold labeled yeast endosomal compartments. Spheroplasts were obtained from the wild type SEY6210 strain (MATalpha ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 mel GAL) as described in the presented protocol. Spheroplasts were then incubated with 4 nmol of positively charged nanogold at 4 °C for 15 min before being transferred to room temperature for 10 (panel A) or 30 min (panel B). Cells were subsequently fixed, processed for cryosectioning[8] before carrying out the silver enhancement reaction on the preparation as described in the presented protocol. A. Early compartments of the yeast endosomal system. In addition to the plasma membrane, a short uptake of the nanogold (5 min) allows the labeling of single vesicles (arrowheads), possibly endocytic vesicles, and of tubular structures (arrow), very likely early endosomes. B. Yeast multivesicular body ultrastructure. A 30 min incubation leads to the labeling of LE/multivesicular bodies (asterisks), but also early endosomes and in part vacuoles (not shown). M, mitochondria; N, nucleus; PM, plasma membrane; V, vacuole. Bar = 200 nm.

Discussion

Immuno-electron-microscopy is a technique that allows combining localization of proteins with the ultrastructural resolution of the carriers and organelles where these proteins reside. This is particularly crucial when studying the yeast endosomal system because its compartments appear as punctuate structures in fluorescence microscopy. It is therefore difficult to distinguish them. For this reason the use of a probe detectable by EM and entering the endocytic route in a time dependent manner is crucial to specifically mark the different endosomes. This type of probe is valuable to assess the functionality of endocytosis[6] and it is an alternative to the lack of antisera detecting protein markers.

The procedure described here exploit positively charged nanogold as a probe to label the endosomal compartments. While cryosectioning requires special equipment and skills, the presented protocol is technically easy and does not require special machines. Therefore it is accessible to any laboratory that would like to use it for its research. However, some precautions have to be taken during the critical step of this procedure, i.e. the silver enhancement reaction. This reaction is highly sensitive to light and therefore all lights (except the red one) in the room have to be turned off and/or covered. As all chemical reactions, the speed of the silver enhancement reaction is influenced by the temperature. To obtain consistent and reproducible results using the same parameters, all solutions and tools have to be carefully preincubated at 24 °C. It is crucial to accurately establish the incubation time for the silver enhancement in the room where this reaction will be routinely performed. Expansion of the nanogold has to be long enough in order to be able to visually detect the gold particle by EM and has to be short enough to avoid the generation of oversized particles, which could cover morphological details. For this reason, it is warmly suggested to perform a pilot experiment when setting up this technique in the laboratory. During this test the nanogold particles have to be silver enhanced for different times to determine the optimal time for the reaction. Of note, the dimensions of the obtained particles cannot be of homogenous size; there will be always some heterogeneity but this has to be kept as minimal as possible, in a range between 5 - 15 nm. This is particularly important if the nanogold uptake procedure will be combined with immuno-gold labelings. Another aspect to be kept in mind when interpreting the results is that pulse-chase labeling experiments are not possible with this method. Consequently the PM and EE will be also labelled in a nanogold uptake aimed to to visualize LE.

It is also possible to see additional applications of the protocol presented here. An option could be to correlate the EM data of nanogold uptake with fluorescent microscopy. FM4-64 is a lipophilic fluorescent marker dye that associates to the PM and, on its way to the vacuole, is passing through the endosomal compartments in a time-dependent manner. Both FM4-64 and nanogold enter the cell in association with lipids and they have probably very similar internalization kinetics[28,29]. As a result both dyes could be used at the same time in correlative light-EM approaches to simultaneously explore the dynamic and the ultrastructure of the endocytic compartments.The presented method can also be used to receptor-mediated trafficking through endocytic system[30]. Monomaleido and mono-*sulfo*-N-hydroxy-succinimido derivatives of the nanogold can be used to covalently bind these particles to proteins[6]. For example cross-linking with the a- or the α-factor can allow following the endocytosis of these two pheromones by their specific receptors.

All together, the described protocol allows to specifically label the organelles of the yeast endosomal system using an endocytosed probe, which can be made detectable by EM. This experimental tool is a valuable approach to study the biogenesis and organization of the yeast endosomal compartments at the ultrastructural level.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors thank René Scriwanek for the assistance with the preparation of the Figure. F.R. is supported by ECHO (700.59.003), ALW Open Program (821.02.017 and 822.02.014), DFG-NWO cooperation (DN82-303) and ZonMW VICI (016.130.606) grants.

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