Observation of Immuno-Labeled Cells at High Resolution Using Soft X-Ray Microscope at Ritsumeikan University SR Center

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Abstract. Mouse fibroblast cell line NIH3T3 cells were labeled with the heavy metal (silver and gold) and observed intracellular structure under an X-ray microscope. Microtubules, Golgi apparatus and early endosomes of NIH3T3 cells were stained with immuno-gold nanoparticles, and immuno-staining was intensified by silver or gold enhancement procedure. Using a transmission soft X-ray microscope beamline (BL12) at Ritsumeikan University SR center, we observed immuno-stained NIH3T3 cells with several wavelengths just below and above oxygen edge ($\lambda = 2.32$ nm). Using this method, cytoskeleton (microtubules) and organelles (Golgi apparatus and early endosomes) were successfully imaged with high resolution. Thus, immuno-gold silver and gold enhancement technique is useful for specific labeling of intracellular structure under an X-ray microscope.

1. Introduction

Although generally labeling is not required for soft X-ray microscopy, a special labeling technique is quite useful. It can enhance contrast at the specific site, which gives us a clue to observe functionally important structures. In this report, we describe results of soft X-ray contrast enhancement of the specific sites of the cells by heavy-metal labeling. As the first step, we labeled mouse fibroblast cell line NIH3T3 cells with the use of silver enhancement [1] or gold enhancement technique [2] for immuno-gold cytochemistry which has been developed for transmission electron microscopy (TEM). NIH3T3 cells were reacted with antibodies against $\alpha$ tubulin (constituent of microtubule), proteins localizing on Golgi apparatus or endosomes and, then with secondary antibody conjugated with small gold particles (~1 nm). The immuno-staining was intensified by depositing silver or gold on the surface of the gold particles by silver or gold enhancement procedure. Using a transmission soft X-ray microscope at BL12, we examined immuno-stained NIH3T3 cells with several wavelengths just below and above the oxygen edge ($\lambda = 2.32$ nm).
2. Materials and Methods

Mouse fibroblast cell line, NIH3T3 cells were cultured in DMEM medium. Cells were attached to polyvinyl formbar (PVF) membrane on Ni grids after poly-L-lysine treatment. The attached cells were fixed in 4% paraformaldehyde in 0.1 M Na-phosphate buffer (pH 7.4) (PB) and washed in PB.

Cells on the membrane were permeabilized in 0.1% Triton-X 100 in PB, and incubated in PB containing 0.005% saponin for blocking. Cells were then treated with mouse monoclonal antibodies (anti-α tubulin, Sigma or anti-GM130, Transduction Lab.) or with rabbit polyclonal antibody (anti-EEA1, Acris) overnight. Then, the cells were washed, and incubated with goat anti-rabbit or anti-mouse IgG that was conjugated to colloidal gold (1.4 nm in diameter) in the blocking solution. Cells were washed with PB, and fixed with 1% glutaraldehyde in PB. After washing, the gold labeling was intensified by using a silver enhancement kit (HQ silver, Nanoprobes) in the dark or by a gold enhancement kit (GoldEnhance, Nanoprobes) in the light. After washing in distilled water, cells were air-dried. X-ray observation was performed at soft X-ray microscope beam line BL12 of Ritsumeikan University SR Center [3].

3. Results and Discussion

Figure 1 shows paraformaldehyde-fixed and air-dried 3T3 cells. 3T3 cells on the PVF membrane showed polygonal shape and extended pseudopodia (arrows in figure 1a and b) as fine projection from them. The fine pseudopodia were clearly imaged under an X-ray microscope. The nucleus was also visible as X-ray dense structure (N in figure 1a and b). X-ray lucent vacuole-like structure was observed in the cytoplasm (arrowhead in figure 1a and b). At present, it remains to be solved whether this vacuole-like structure corresponds to some cell structure or is an artifact produced by the fixation and air-dry processes. In unstained cells, we could not distinguish other cellular structures. Next, we observed 3T3 cells immuno-stained with immuno-gold and silver or gold enhancement technique using antibodies against microtubule, Golgi apparatus and endosomes.

Microtubule is tubular structure about 25 nm in diameter, and plays important roles in cell shape regulation, in cell division and in intracellular traffic as one of cytoskeletons of cells. Figure 2 shows tubulin-stained 3T3 cells. Tubulin is the constituent protein of microtubules. Under light microscope, silver enhancement procedure makes stained-tubulin brown (figure 2a, dark color). X-ray microscope imaged many microtubules as fibers radically expanding from the centrosome around the nucleus towards the cell surface (figure 2b). As a result of adjusting the image contrast based on each microtubule, structures of the cytoplasm reconstructed by stitching of small parts were somewhat obscure, but extension of microtubules is clearly visible (figure 2b, white arrows). This result shows that cellular structure (such as microtubule; 25 nm in diameter) which has smaller size than the resolution of the X-ray microscope can be imaged by the immuno-labeling by heavy metal.

Golgi apparatus is an organelle, playing important roles as a center of intracellular traffic and locates near the nucleus. Figure 3 shows silver enhancement of immuno-gold staining of 3T3 cells using antibody against GM130 (Golgi matrix protein 130), which was localized on the Golgi apparatus. Under a light microscope, Golgi apparatus was observed near the nucleus in brown color (figure 3a, dark color). As shown in figure 3b, Golgi apparatus was imaged as X-ray dense structure though the image was somewhat obscure by overlapping with the nucleus. Golgi apparatus was also visualized as X-ray dense structure by gold enhancement procedure of immuno-gold staining of GM130 (data not shown).

The endosome is an organelle, playing important roles in the traffic and the sorting in endocytosis, and consists of early endosome and late endosome. Figure 4 shows gold enhancement for EEA1 (early endosome antigen 1), which was localized on early endosomes. Early endosomes were imaged under X-ray microscope as small dot-like structures (figure 4b). We could only distinguish limited numbers of early endosomes in a cell. This suggests immuno-labeling by this antibody was not sufficiently strong to stain all of the early endosomes under an X-ray microscope. Early endosomes were also observed as small dot-like structures by silver enhancement for EEA1 (data not shown).
4. Summary
It was shown that immuno-gold silver enhancement technique and immno-gold enhancement technique is useful for specific labeling of intracellular structure under an X-ray microscope. Using this method, cytoskeleton (microtubules) and organelles (Golgi apparatus and early endosomes) were successfully observed with high resolution. The improvement of X-ray contrast may be achieved by examining antibodies and staining procedure, and enable us much more intimate analyses of the cell structure under an X-ray microscope.

Figure 1. A NIH3T3 cell. (a) Light microscopic image, (b) X-ray microscopic image at 2.4 nm. Exposure time was 120 s. An arrow shows a projection of the cell. An arrowhead indicates vacuole-like structure. N; nucleus, scale bar is 10 μm.

Figure 2. A NIH3T3 cell immuno-labeled by anti-tubulin antibody using immunogold-silver enhancement technique. (a) Light microscopic image, (b) X-ray microscopic image at 2.3 nm. Exposure time was 60 s. The rectangular region of (a) is shown in (b). White arrows in (b) show microtubules. Arrowheads show cell surface. N; nucleus, scale bar is 10 μm.

Figure 3. A NIH3T3 cell immuno-labeled by anti-GM130 (Golgi protein) antibody using immunogold-silver enhancement technique. (a) Light microscopic image, (b) X-ray microscopic image at 2.3 nm. Exposure time was 60 s. Arrows show Golgi region. An arrowhead shows a dot produced artificially. It is not meaningful contrast, and does not correspond to the any cellular structures. N; nucleus, scale bar is 10 μm.

Figure 4. A NIH3T3 cell immuno-labeled by anti-EEA1 (endosome protein) antibody using immunogold-silver enhancement technique. (a) Light microscopic image, (b) X-ray microscopic image at 2.3 nm. Exposure time was 60 s. The rectangular region of (a) is shown in (b). An arrow shows an endosome. N; nucleus, scale bar is 10 μm.

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