Observation of Immuno-Labeled Cells at High Resolution Using Soft X-Ray Microscopy

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Abstract

The soft X-ray microscopy is one of the most powerful tools to observe living cells at high resolution. In this report, we labeled mouse fibroblast cell line NIH3T3 cells 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 at BL12, we observed immuno-stained NIH3T3 cells with several wavelengths just below and above oxygen edge (= 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.

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1. Introduction

X-ray microscopy today uses electromagnetic radiation wavelengths from 45 nm to 0.04 nm. Because of its short wavelength, sub-optical spatial resolution can be obtained. Several different contrast mechanisms can be used to form images, thereby making X-ray microscopes useful for probing different properties of matter.

Soft X-ray microscopes can be used to observe hydrated whole cells [1-5]. Using the X-ray transmissible wavelength region where organic material absorbs approximately an order of magnitude more strongly than water (so-called "water window") [6], X-ray imaging will be invaluable for studying hydrated organic matter with high resolution without any chemical contrast enhancement agents.

The soft X-ray microscopy may become the most powerful tool to observe living cells at high resolution and to study function of sub-cellular structure such as organelles or cytoskeleton in near future. 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 [7] or gold enhancement technique [8] for immuno-gold cytochemistry which has been developed for transmission electron microscopy (TEM)., NIH3T3 To this end, cells were reacted with antibodies against 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 (λ = 2.32 nm).

2. Materials and methods

2.1 Sample preparation

Cell culture and fixation

Fibroblast cell line, NIH3T3 cells were cultured in DMEM medium. Cells were attached to polyvinyl formbar (PVF) membrane on nickel grids for 2 or 3 h. To ensure the attachment of the cells, PVF membrane was previously incubated with aqueous solution of poly-L-lysine (5μg/ml) for 1min. and rinsed with distilled water 3 times. The attached cells were fixed in 4% paraformaldehyde in 0.1 M Na-phosphate buffer (pH 7.4) (PB) for 30 min. and washed three times in PB.

Immuno-labeling.

Cells on the membrane were permeabilized in 0.1% triton-X100 in PB for 30 min., and

incubated in PB containing 0.005% saponin, 10% bovine serum albumin (BSA), 10% normal goat serum, and 0.1% cold water fish skin gelatin for blocking for 30 min. Cells were then treated with mouse monoclonal antibody against α tubulin (diluted x500, Sigma), mouse monoclonal antibody against GM130 (diluted x200, Transduction laboratory), or rabbit antibody against EEA1 (diluted x200, Acris) in the blocking solution, overnight. Then, the cells were washed in PB containing 0.005% saponin for 10 min six times, and incubated with goat anti-rabbit or anti-mouse IgG that was conjugated to colloidal gold (1.4 nm diameter) in the blocking solution for 2 hours. Cells were washed with PB for 10 min six times, and fixed with 1% glutaraldehyde in PB for 10 min. After washing, the gold labeling was intensified by using a silver enhancement kit (HQ silver, Nanoprobes, NY) for 6 min at 20°C in the dark or by a gold enhancement kit (GoldEnhance, Nanoprobes, NY) for 5 min at 20°C in the light. After washing in distilled water, cells were air-dried.

2.2 Soft X-ray microscopy

X-ray microscopic observation was performed at beamline BL12 [9-10]. The optical element of the X-ray microscope is zone plates used for imaging or focusing. The outermost zone width and aspect ratio define the achievable spatial resolution and diffraction efficiency, respectively. The achieved resolution is c.a. 70nm judging from the knife-edge estimation (20 % -80 %). The expected energy resolution (E/ΔE) is about 160. The size of view filed is about 10 μm × 10 μm. The magnified image is recorded on a peltier-cooled, back-illuminated X-ray CCD camera with 512 ×512 pixels.

3. Results and Discussion.

3.1 Unstained cell.

Fig.1 shows paraformaldehyde-fixed and air-dried 3T3 cells. 3T3 cells on the PVF membrane showed polygonal shape and extended pseudopodia (arrows in Fig. 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 Fig. 1a and b). X-ray lucent vacuole-like structure was observed in the cytoplasm (arrowhead in Fig. 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.

3.2 Immuno-stained cells

Next, we observed 3T3 cells immuno-stained with immuno-gold and silver or gold enhancement technique using antibodies against microtubule, Golgi appratus and endosomes.

3.2.1 Microtubule. 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. Fig. 2 shows tubulin-stained 3T3 cells. Tubulin is the constituent protein of microtubules. Under light microscope, silver enhancement procedure makes stained-tubulin brown (Fig 2a, dark color). X-ray microscope clearly imaged many microtubules as fibers radially expanding from the centrosome around the nucleus towards the cell surface (Fig. 2b). 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 imuno-labeling by heavy metal.

3.2.2 Golgi apparatus. Golgi apparatus is an organelle, playing important roles as a center of intracellular traffic and locates near the nucleus. Fig. 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 appratus. Under a light microscope, Golgi apparatus was observed near the nucleus in brown color (Fig 3a, dark color). As shown in Fig 3b, Golgi apparatus was imaged as X-ray dense structure though the image was somewhat obscure by overlapping with the nucleus. Fig. 4 shows gold enhancement of immuno-gold staining of GM130. Under a light microscope, Golgi apparatus was stained blue (Fig 4a, dark color). The contrast of Golgi apparatus was also strengthened under an X-ray microscope.

3.2.3 Endosomes. The endosome is an organelle, playing important roles in the traffic and the sorting in endocytosis, and consists of early endosome and late endosome. Fig. 5 shows silver 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 (Fig, 5b). Fig. 6 shows gold enhancement for EEA1. Early endosomes were also observed as small dot-like structures. 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.

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.

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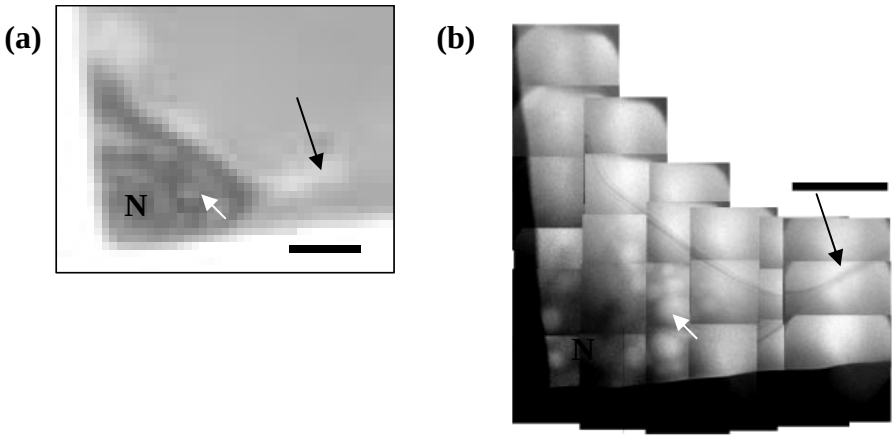

Fig. 1. Light microscopic and X-ray microscopic images of a NIH3T3 cell. (a) light microscopic image, (b) X-ray microscopic image at 2.4 nm. Exposure time was 120 s. An arrow shows a projection of the cell. An arrowhead indicates vacuole-like structure. N; nucleus, Scale bar is 10 μm.

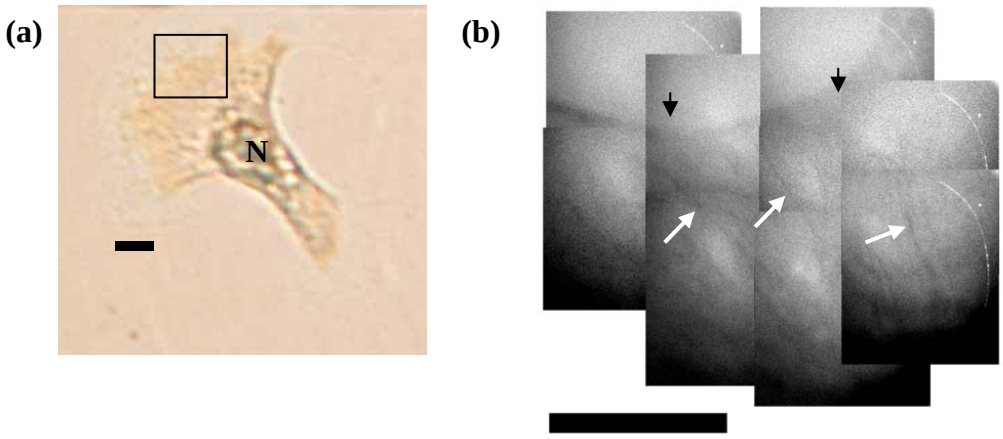

Fig. 2. Light microscopic and X-ray microscopic images of a NIH3T3 cell immuno-labeled by anti-tubulin antibody using immunogold-silver enhancement technique. (a) light microscopic image, (b) X-ray microscopic image at 2.3 nm. Exposure time was 60 s. The rectangular region of Fig. 2a is shown in Fig. 2b. Arrows in Fig. 2b show microtubules. Arrowhead show cell surface. N; nucleus, Scale bar is 10 μm.

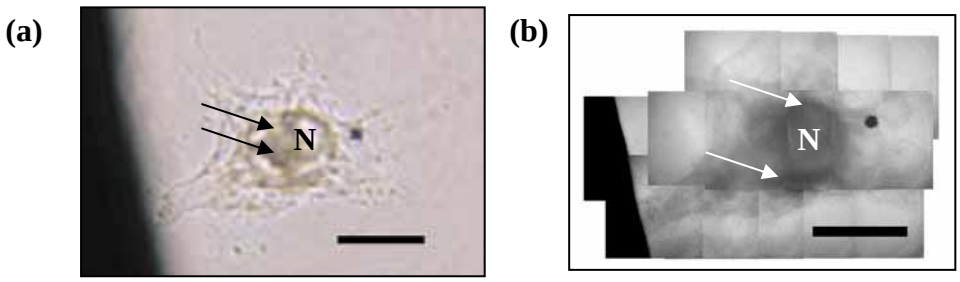

Fig. 3. A NIH3T3 cell immuno-labeled by anti-GM130 (Golgi protein) antibody using immunogold-silver enhancement technique. (a) light microscopic image, (b) X-ray microscopic image at 2.3 nm. Exposure time was 60 s. Arrows show Golgi region. Arrowheads show a dot produced artifactually. N; nucleus, Scale bar is 10 μm.

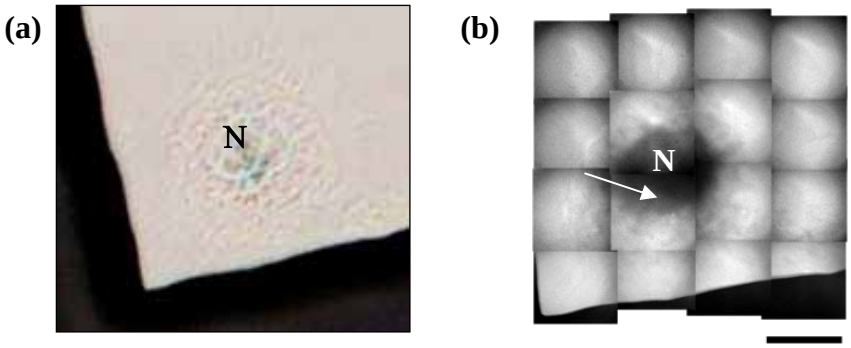

Fig. 4. A NIH3T3 cell immuno-labeled by anti-GM130 (Golgi protein) antibody using immunogold-gold enhancement technique. (a) light microscopic image, (b) X-ray microscopic image at 2.3 nm. Exposure time was 60 s. An Arrow shows Golgi region. N; nucleus, Scale bar is 10 mm.

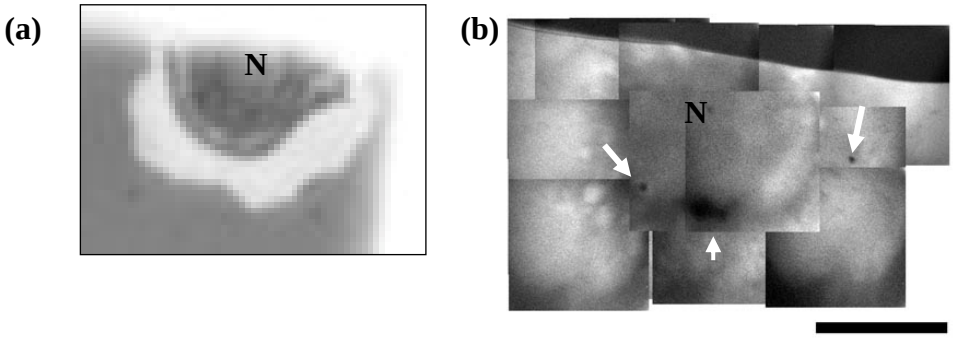

Fig. 5. A NIH3T3 cell immuno-labeled by anti-EEA1 (endosome protein) antibody using immunogold-silver enhancement technique. (a) light microscopic image, (b) X-ray microscopic image at 2.4 nm. Exposure time was 60 s. Arrows show early endosomes. An arrowhead shows artifact. N; nucleus, Scale bar is 10 μm.

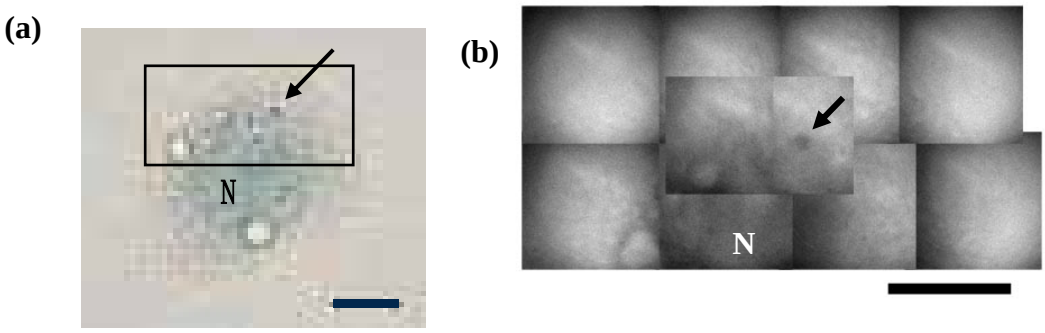

Fig. 6. A NIH3T3 cell immuno-labeled by anti-EEA1 (endosome protein) antibody using immunogold-silver enhancement technique. (a) light microscopic image, (b) X-ray microscopic image at 2.3 nm. Exposure time was 60 s. The rectangular region of Fig. 6a is shown in Fig. 6b.An arrow shows an endosome. N; nucleus, Scale bar is 10 μm.

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