Combination of a cryosectioning method and section scanning electron microscopy for immuno-scanning electron microscopy

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ABSTRACT

We describe a novel immuno-scanning electron microscopy (SEM) technique that combines both Tokuyasu's cryosectioning and section SEM methods. In this technique, semithin cryosections, cut according to the Tokuyasu method, were adhered to glass microscope slides, immunostained for bio-molecules of interest and observed by confocal laser scanning microscopy. The same sections were subsequently embedded in epoxy resin and ultrathin sections were cut on an ultramicrotome. These were then observed by SEM using a backscattered electron detector. Correlation between immunofluorescence and SEM images was performed in the same area of the cryosection. Immuno-SEM was also performed using a FluoroNanogold-labeled secondary antibody. This novel immuno-SEM method can provide ultrastructural information of cell organelles in relation to associated molecules, such as Golgi- and ER-associated proteins. This novel immuno-SEM technique has the potential to be widely used.

The ultrathin cryosection technique developed by Dr. Tokuyasu is a valid method for the immunocytochemical detection of subcellular antigens at the electron microscopic level (6–8). In this cryosectioning technique, specimens are prepared in a high molar sucrose solution or in a mixture of polyvinylpyrrolidone (PVP) and high molar sucrose to prevent ice-crystal formation. These cryoprotectants result in both good preservation of ultrastructure and high antigenicity in cells; sections cut according to the procedure have better antigen accessibility compared with sections prepared from specimens embedded in resins such as epoxy resin and LR white resin.

More recently, we have introduced a novel imaging method for observing resin-embedded sections by scanning electron microscopy (SEM) (1, 2). In this technique, semithin or ultrathin sections adhered

to a glass slide are stained with both uranyl acetate and lead citrate, and observed with backscattered electron (BSE)-mode SEM at a low accelerating voltage. Using this method, we can obtain ultrastructural images similar to conventional TEM of ultrathin biological sections, because BSE imaging of resin-embedded sections at a low accelerating voltage provides information very near the surface of the section (2).

In the present study, we combine the Tokuyasu method of semithin cryosectioning with the section SEM technique to develop a novel immuno-SEM method. Using FluoroNanogold (FNG)-labeled secondary antibodies, correlation between immunofluorescence microscopy and immuno-SEM is performed without a complicated procedure. Furthermore, our SEM method can provide ultrastructural information on subcellular structures with respect to associated functional molecules.

MATERIALS AND METHODS

Antibodies. A rabbit polyclonal anti-rat luteinizing hormone (LH) antibody (kindly provided by Dr.

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Matozaki, Gunma University) was used to identify gonadotropes. A mouse monoclonal antibody against Binding immunoglobulin protein (BiP), an ER chaperon protein, was purchased from BD Biosciences (San Jose, CA). A sheep polyclonal anti-TGN 38 antibody (AbD Serotec, Oxford, UK) was also used for immunofluorescence microscopy. Secondary antibodies conjugated with fluorescent dyes (Alexa Fluor 488-labeled anti-rabbit IgG; Alexa Fluor 647-labeled anti-mouse IgG) were purchased from Invitrogen (Carlsbad, CA, USA). A biotinylated secondary antibody (anti-sheep IgG; purchased from Abcam, Cambridge, UK) and Alexa Fluor 594-labeled streptavidin FNG (Invitrogen) were also used for immunofluorescence microscopy.

Animals. Male Wistar rats, aged 8 weeks old, were used. Animal experiments were approved by the Committee of the Ethics on Animal Experimentation of Kagoshima and Asahikawa Medical Universities in accordance with their Guidelines for Animal Experimentation (no. MD15028 and no. 02-006).

Immunofluorescence microscopy of semithin cryosections. Under deep anesthesia with pentobarbital sodium, animals were perfused through the ascending aorta with physiological saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion fixation, excised pituitaries were cut into small pieces and further fixed in the same fixative for 24 h at 4°C. They were then immersed sequentially in 0.4, 0.9, 1.5, and 2.3 M sucrose, and finally in a mixture of 20% PVP and 1.84 M sucrose solution for cryoprotection (3 h in each solution, at 4°C). Specimens were then put on a sample pin, quickly frozen in liquid nitrogen and placed in a cryochamber (EM FC7; Leica Microsystems, Nussloch, Germany) attached to an ultramicrotome (Ultracut EM UC7; Leica Microsystems). Semithin cryosections (1 µm thick) were then cut from the frozen specimen in the low-temperature sectioning system at -65 to -75°C using a diamond knife (Diatome, Biel, Switzerland) (Fig. 1a). Cryosections were picked up with a 2.3 M sucrose droplet on a 2 mm diameter wire loop and mounted on glass microscope slides. Subsequently, they were rinsed with phosphate buffered saline (PBS) for 30 min at 4°C and then blocked with 2% normal donkey serum for 30 min at 20°C, followed by incubation with a mixture of primary antibodies of different species (a rabbit polyclonal anti-rat LH antibody, a mouse monoclonal anti-rat BiP antibody and a sheep polyclonal anti-TGN 38 antibody) for 12 h at 4°C. Sec-

tions were then rinsed with PBS (3 min, three times) and incubated with a mixture of secondary antibodies (Alexa Fluor 488-labeled anti-rabbit IgG, Alexa Fluor 647-labeled anti-mouse IgG and biotin-labeled anti-sheep IgG) for 1 h at 20°C, followed by further rinses in PBS (3 min, three times). They were then incubated with streptavidin-labeled FNG conjugated with Alexa 594 for 1 h at 20°C and then washed with PBS (3 min, three times). After coverslips were mounted on sections using SlowFade® Diamond (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, Mo, USA), immunostained sections were observed by confocal laser scanning microscopy (CLSM) (LSM 700; Carl Zeiss Microscopy GmbH, Jena, Germany) (Fig. 1b).

SEM observation of semithin cryosections. Cryosections observed by CLSM were washed with PBS until the mounting medium was completely removed and were then fixed with 2% glutaraldehyde in 0.1 M PB for 1 h at 4°C (Fig. 1c). Subsequently, they were rinsed with 0.1 M PB for 10 min and incubated in a gold enhancing solution (GOLDENHANCE™ EM; Nanoprobes, NY, USA), for 30 s at 20°C to enlarge the labeled nanogold particles (Fig. 1d). After sections were further stained with 2% OsO₄ containing 1.5% potassium ferrocyanide (reduced osmium) for 1 h, 1% aqueous uranyl acetate for 1 h, and 1% aqueous lead citrate for 5 min (Fig. 1e), they were dehydrated in a graded ethanol series and embedded in epoxy resin at 60°C for 24 h (Fig. 1f). The resin blocks were then peeled off the glass slides, trimmed to a trapezoidal shape, and ultrathin sections (100 nm thick) were cut with an ultramicrotome using a diamond knife (Micro Star, Tokyo, Japan) (Fig. 1g). Ultrathin sections were attached to a glass slide by heating on a hot plate for 30 min at 60°C (Fig. 1h), coated with carbon (1 nm thickness) using a carbon coater (VC-100; Vacuum Device, Ibaraki, Japan) (Fig. 1i) and observed in a semi-in-lens type field emission scanning electron microscope (SU-70; Hitachi, Tokyo, Japan) using the BSE detector with an accelerating voltage at 2.5 kV (Fig. 1j). Finally, correlative microscopy was performed by observing the same area in immunofluorescence and SEM images (Fig. 1k).

RESULTS AND DISCUSSION

Immunofluorescence images of gonadotropes in the rat pituitary gland are presented in Fig. 2a–d. The semithin cryosections were immunocytochemically stained with LH (colored yellow; Fig. 2a), the ER

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Fig. 1 Outline of the combination of cryosectioning and section SEM methods for immuno-SEM. (a) Semithin cryosections are cut using an ultramicrotome equipped with a cryo-chamber. (b) A cryosection on a glass microscope slide is immuno-cytochemically stained with primary antibodies and a mixture of AlexaFluor labeled- and FluoroNanogold labeled-secondary antibodies. The section is observed with confocal laser scanning microscopy. (c) The section is fixed with 2% glutaraldehyde (GA) for 1 h (d) and then incubated in a gold enhancing solution to enlarge nanogold particles. (e) The specimen is then heavy metal stained with reduced osmium solution for 1 h, uranyl acetate solution for 1 h and lead citrate solution for 5 min. (f) The cryosection is then dehydrated and embedded in epoxy resin. The resin block on the glass slide is peeled off and trimmed under a stereoscopic microscope. (g) Ultrathin sections of the resin-embedded cryosection are cut using an ultramicrotome with a diamond knife. (h) The sections are then adhered to a glass slide by heating on a hot plate (i) and then coated with carbon. (j) The sections are then observed with SEM using a BSE detector. (k) Correlation between immunofluorescence and SEM images is performed by observation of the same area. High resolution imaging is also performed by observing the localization of gold particles.

Fig. 2 Application of the immuno-SEM method. A semithin cryosection of the rat pituitary gland was immunocytochemically stained with sheep polyclonal anti-LH (yellow pseudocolor, **a**), mouse monoclonal anti-BiP (magenta pseudocolor, **b**) and sheep polyclonal anti-TGN38 (green pseudocolor, **c**) antibodies. Nuclei were labeled with DAPI (blue pseudocolor, **d**). The merged image is shown in (**d**). (**e**) Ultrathin section image of the LH-positive cell shown in (**d**) was recorded by backscattered electron (BSE)-mode SEM. A black and white reversal image is shown. N: nucleus. (**f**) The immunofluorescence signals are merged on the BSE image shown in (**e**). The immunocytochemical location of LH (yellow), BiP (magenta), TGN38 (green) and DAPI (blue) corresponds to secretory granules, the ER, the Golgi apparatus and the nucleus (N), respectively. Higher magnification image of the boxed area in (**f**) is enlarged and shown in (**g**). The fluorescence image of TGN38 labeled with FluoroNanogold (green) is overlaid on the BSE image. The original BSE image in (**g**) is shown in (**h**). The localization of gold particles (*i.e.*, TGN38) is clearly seen (arrows) on *trans*-cisterna of the Golgi apparatus. N: nucleus, M: mitochondria, Sg: secretory granules, asterisks: the ER. Bars: (d) 10 µm; (f) 5 µm; (h) 0.5 µm.

chaperon protein, BiP (colored magenta; Fig. 2b) and the Golgi associated protein, TGN38 (colored green; Fig. 2c). Nuclei were stained with DAPI (colored blue; Fig. 2d). A merged image is shown in Fig. 2d. Dilated cisternae of the rough ER were distributed throughout the cytoplasm, and a ring-shaped Golgi apparatus was clearly visible. Swelling of the ER lumen is often recognized in gonadotropes of control rats. After observation by CLSM, the cryosection was embedded in epoxy resin and cut into several ultrathin sections using a diamond knife. They were then adhered to a glass microscope slide and observed by SEM using a BSE detector. A black and white reversed BSE image of the same region in the immunofluorescence image of Fig. 2d is presented in Fig. 2e. Reversed BSE images of ultrathin sections resemble TEM images of biological specimens (2).

The immunofluorescence images shown in Fig. 2d were overlaid on the ultrathin section image (Fig. 2f). By correlating the fluorescence and BSE images, the localization of molecules was vividly recognized on the ultrathin section; LH, Bip and TGN38 were located on secretory granules, the ER and the Golgi apparatus, respectively.

The ultrastructure of secretory granules, the ER (asterisks in Fig. 2g), the Golgi apparatus and mitochondria was clearly observed by SEM (Fig. 2g). The signal intensity of BSE depends on the atomic number of the staining material; staining with heavy metals such as osmium, uranium and lead enhances BSE images of subcellular structures. In Fig. 2g, the immunofluorescence and SEM image were superimposed to precisely observe the localization of the anti-TGN38 antibody. However, the location of the molecule was unclear in the merged image. This is because when the immunofluorescence image is enlarged to the electron microscopic resolution level, exudation of the fluorescence dye occurs. To overcome this problem, the primary antibody (*i.e.*, TGN38) was labeled with a secondary antibody conjugated with fluorescent dye and nanogold. Using this secondary antibody, the localization of the Golgi protein can be clearly demonstrated by SEM in the ultrathin section of the Epon-embedded cryosection; the gold particles were visualized on the *trans*-cisterna of the Golgi apparatus (arrows in Fig. 2h).

FNG is a bi-functional labeling probe consisting

of both a fluorescent dye and a 1.4 nm gold particle conjugated to an antibody Fab' fragment, IgG or streptavidin (3–5). As FNG can be detected by both fluorescence microscopy and TEM or SEM, this secondary antibody has been used for correlative microscopy (2, 4). As the signal intensity of BSE depends on the average atomic number of the specimen, enhancement by the nanogold particles (atomic number: 79) was clearly recognized by BSE-mode SEM.

The present study introduces a novel immuno-SEM technique developed by combining a cryosectioning method and section SEM. The relationship between the localization of molecules and the ultrastructure of subcellular structures was clearly demonstrated using immuno-SEM. This technique is applicable to investigate the functional morphology of many different several kinds of tissue.

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