Three-dimensional high voltage electron microscopy of thick biological specimens

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Received 13 July 1999; received in revised form 10 January 2000; accepted 10 January 2000

Abstract

The procedures recently developed in our laboratory to observe three-dimensional structures of cell organelles in thick biological specimens by means of high voltage electron microscopy are reviewed. Thick biological specimens such as whole mount cultured cells seeded and grown on grid meshes in culture vessels or thick sections cut from embedded tissues and stained by histochemical reactions can be readily observed three-dimensionally by high voltage transmission electron microscopy at 400–1000 kV. Cultured cells used were both primary cultures from animal tissues and established cell lines maintained in our laboratory. The livers of adult Wistar rats were isolated by collagenase perfusion, and hepatocytes were suspended in a Leibovitz medium, incubated in a CO\textsubscript{2} incubator in a humidified atmosphere containing 5\% CO\textsubscript{2} in air at 37°C for a few days. Established cell lines, CHO-K1 cells, were cultured in Ham’s F12 medium, while HeLa cells were cultured in Eagle’s MEM under the same condition. Some of the cells were cultured under experimental conditions such as hepatocyte culture in the medium containing peroxisome proliferating agents such as clofibrate or bezafibrate and some of them were labeled with \textsuperscript{3}H-thymidine, \textsuperscript{3}H-uridine, \textsuperscript{3}H-labeled precursors and \textsuperscript{14}C-bezafibrate. Also some cells were incubated in medium containing HRP to induce pinocytosis. All the whole mount cultured cells on grid meshes were prefixed in buffered 2.5\% glutaraldehyde, stained with various histochemical reactions and postfixed in 1\% osmium tetroxide. The histochemical reactions used were glucose-6-phosphatase (G-6-Pase), thiamine pyrophosphatase (TPPase), cytochrome oxidase, acid phosphatase (AcPase), DAB, ZIO, PA–TCH–SP reactions and radioautography was performed after labeling with radiolabeled compounds. The whole mount cultured cells were dried in a critical point dryer and were observed with JEOL JEM-4000EX or Hitachi H-1250M high voltage electron microscopes at 400–1000 kV. By tilting the specimens’ stereo-pair micrographs were recorded and they were observed with stereoscopes. Rat liver, mouse intestine and pancreas tissues, fixed and stained as above, were embedded in Epoxy resin, thick sectioned at 1–2 \textmu m and were observed as for the whole mount cultured cells at 1000 kV. Stereo-pairs were further analyzed with an image analyzer JEOL JIM-5000 (JEOL, Tokyo, Japan), producing two contour lines plotted from the micrographs at a thickness of 0.2 \textmu m and were observed with anaglyph type glasses, demonstrating the depth or heights of respective cell organelles. The results show that whole mount cultured cells and thick sections stained with histochemical reactions reveal cell organelles corresponding to marker enzymes, such as G-6-Pase in endoplasmic reticulum, TPPase and ZIO in Golgi apparatus, cytochrome oxidase in mitochondria, AcPase in lysosomes, DAB in peroxisomes and pinocytotic vesicles, PA–TCH–SP in secretory granules, \textsuperscript{3}H-thymidine and \textsuperscript{3}H-uridine in nuclei, \textsuperscript{3}H-animo acids in endoplasmic reticulum and secretory granules, \textsuperscript{14}C-bezafibrate around ER and peroxisomes. The ultrastructure of these cell organelles as well as the structural relationship between them can be demonstrated three-dimensionally with stereo-pair images. Overall, these procedures are useful for analyzing stereologically the ultrastructure of cell organelles in cells and tissues. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: High voltage electron microscopy; Histochemistry; Three-dimensional observation; Cell organelles; Thick specimens; Tissue culture

1. Introduction

Thick biological specimens such as whole mount cultured cells or thick sections from resin embedded tissues can be successfully observed three-dimensionally by high voltage transmission electron microscopy at 400–1000 kV. Observation on whole mount cells in culture by electron microscopy was first carried out by Porter et al. (1945) when they tried to observe thin cytoplasmic processes of cultured cells without sectioning at the initial stage of electron microscopy applied to biological materials because the ultramicrotomes which were developed later were not available at that time and there was no other way to obtain thin specimens.
However, once ultramicrotomes were developed and ultrathin sections easily prepared for electron microscopic observation, such whole mount methods cultured cells were not used during the period from the 1950s to the early 1970s. In the late 1970s they were again tried by Buckley and Porter (1975) to observe the cytoskeletons of whole mount cultured cells by high voltage electron microscopy (HVEM) at the accelerating voltage of 1000 kV. Wolosewick and Porter (1975, 1976, 1977, 1979) further studied cytoskeletons of whole mount cultured cells in vitro. They observed whole mount cultured cells without any histochemical reactions but only drying them with a critical point dryer which was formerly used in preparing specimens for scanning electron microscopy. This period from the late 1970s to the early 1980s when Porter et al. observed the cytoskeletons of whole mount cultured cells should be regarded as the second period to employ such thick preparations by high voltage electron microscopy.

Since the recent development of very high voltage electron microscopes at accelerating voltages of around 1000 kV, which are very expensive tools to purchase, as well as intermediate high voltage electron microscopes at accelerating voltages of 300–400 kV, which are less expensive and commercially easily available in conventional laboratories, many papers dealing with the ultrastructure of various biological materials, cells and tissues have been published (Glauert, 1979; Nagata, 1984). Microorganisms such as trypanosoma (De Souza and Benchimol, 1984) or leptospira and borrelia (Goldstein et al., 1996) were observed by HVEM. The ultrastructure of free cells obtained from animals and men such as normal and sickled human erythrocytes (Wise et al., 1981), sperm axonema (Baccetti et al., 1985) and mast cell degranulation (Tasaka et al., 1990) were observed by HVEM. Stereo-visual ultrastructure of cells and tissues in many organs obtained from mammalian and lower animals using thick sections were also observed three-dimensionally by HVEM such as sarcoplasmic reticulum in the skeletal muscle fibers of rat and mice stained with silver impregnation (Hayashi and Nagata, 1991), the liver, pancreas, kidney and salivary glands of rats and mice stained with acid phosphatase to demonstrate nematolysosomes (Sakai et al., 1992), isolated glomeruli of Wistar rats and the extracellular matrices of the kidney.
obtained from human patients suffering from renal diseases (Shikata et al., 1992), mouse taste buds (Kinnamon et al., 1993; Royer and Kinnamon, 1994) and collagen fiber bundles in mouse palatine mucosa (Watanabe et al., 1999). The three-dimensional structure of the processes of neurons and neuroglial cells in the nervous and sensory systems were also extensively studied by many authors in neurology, such as the processes of neurons and neuroglial cells (Hama, 1989, 1994), synaptic junctions in the rat cereberum (Igarashi et al., 1998), retinal neuronal cells in fishes (Hidaka et al., 1993; Hidaka and Hashimoto, 1995). The intercellular adhesion molecule-1 in endothelial cells in the blood brain barrier stained immunohistochemically was also observed by HVEM (Lossinsky et al., 1999).

On the contrary, whole mount cultured cells as was reported by Porter and his associates were recently applied to various biological materials with histochemical staining. The open canalicular system in the blood platelets of men stained with lectin (Kawakami and Hirano, 1985) and the cytoskeletons in cultured glioma cells were demonstrated by immunocytochemistry (Taomoto, 1991) using HVEM. Radioautograms prepared from whole mount cultured cells which were previously labeled with radioactive compounds were also observed three-dimensionally by HVEM (Nagata et al., 1994).

In order to demonstrate the three-dimensional structure of cell organelles in cells and tissues when observed by HVEM, we have developed methods to prepare thick biological specimens either from whole mount cultured cells or thick sections obtained from embedded tissues, which were subsequently stained with various histochemical reactions for specific cell organelles, corresponding to the marker enzymes such as G-6-Pase for endoplasmic reticulum, TPPase for Golgi apparatus, cytochrome oxidase for mitochondria, AcPase for lysosomes, DAB in peroxisomes and pinocytotic vesicles, specific histochemical reactions such as ZIO for the Golgi apparatus and PA-TCH-SP for secretory granules, RI-labeled compounds in radioautograms at the synthetic sites of macromolecules such as DNA, RNA, proteins, glucides and lipids or at the binding sites of receptors can also be observed by HVEM at accelerating voltages of 400–1000 kV (Nagata, 1984, 1995, 1997, 1998, 1999; Nagata and Usuda, 1984; Nagata et al., 1977, 1986, 1987, 1990). The procedure is very useful in analyzing stereological...
ultrastructure in cells and tissues. This paper reviews the methods developed in our laboratory to prepare thick biological specimens and presents results obtained by these procedures.

2. Preparation of specimens

2.1. Cells and tissues

Whole mount cultured cells, and the thick sections obtained from experimental animals which were embedded and sectioned, were used.

2.1.1. Whole mount cultured cells

The cultured cells used were both primary culture and established cell lines. For primary culture, hepatocytes were prepared from the livers of normal male adult Wistar rats, weighing about 150 gm, perfused with collagenase (Type IV, Sigma, St. Louis, MO, USA), according to the perfusion technique of Berry and Friend (1969) and free hepatocytes were isolated from other cell types by centrifugation at 3000 rpm for 5 min. The hepatocytes were suspended in a Leibovitz L-15 medium (Nissui, Tokyo, Japan) supplemented with 10% newborn bovine serum (Flow Laboratories, Stanmore, NSW, Australia) and plated at a concentration of $1.0 \times 10^6$ viable cells per 35 mm plastic Petri dish (Falcon Plastic, CA, USA) which contained formval and collagen coated gold meshes (150 meshes, Nisshin EM Co., Tokyo, Japan). Cells were cultured in a CO$_2$ incubator (Tabai, Tokyo, Japan) at 37°C, in a humidified atmosphere containing 5% CO$_2$ in air, for a few days. Some of the cultured hepatocytes were further cultured for a few days in a medium containing 0.2 mM clofibrate, 2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester, or 0.5 mM bezafibrate to induce peroxisome proliferation. Some of the hepatocyte cultures were labeled with radioactive compounds for varying times under the same condition as above to prepare radioautograms. Other cells from any experimental animals can be used to demonstrate various cell organelles.

The established cell lines, both HeLa S3 cells and CHO-K1 cells, maintained in our laboratory, were used. HeLa S3 cells were subcultured in Eagle’s MEM (Nissui, Tokyo, Japan) supplemented with 10% bovine serum (Flow Laboratories, Stanmore, NSW, Australia) onto collodion coated copper meshes in Petri dishes under the same condition as hepatocytes. CHO-K1 cells were trypsinized, suspended in Ham’s F12 medium (Nissui, Tokyo, Japan) supplemented with 10% newborn bovine serum (Flow Laboratories, NSW, Australia) and seeded onto formval coated gold meshes (Nisshin EM, Tokyo, Japan) in Petri dishes (Falcon Plastic, CA, USA) and incubated in a CO$_2$ incubator. Some cells were cultured in Ham’s F12 medium containing HRP (horse radish peroxidase, 1 mg/ml) for 48 h to induce pinocytic vesicles. All the whole mount cultured cells on meshes were rinsed in...
Hanks’ solution for a few minutes, fixed in 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer for 1 h, washed three times in the same buffer, except for radioautography which was carried out after the osmium fixation. After the histochemical reactions, the grid meshes were dehydrated in graded ethanol series, dried in critical point dryers (Hitachi HCP-1 or JEOL JCPD-5, Tokyo, Japan) for electron microscopy.

2.1.2. Thick sections

We used both rat and mouse liver and pancreatic tissues for enzyme histochemistry as well as for radioautography. The rat livers, obtained from either normal or DEHP treated Wistar rats which were fed with 2% DEHP (diethyl-hexyl phthalate, a peroxisome proliferator) containing diet for 1 or 2 weeks, were prefixed in 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer for 1 h, treated with the DAB reaction (Angermüller and Fahimi, 1981) and postfixed in 1% osmium tetroxide in the same buffer, embedded in Epok 812 resin (Oken Co, Tokyo, Japan). Semi-thin and thick sections at 0.1, 0.2, 0.5, 1.0 and 2.0 μm thick were cut, picked up onto collodion coated copper grids (Nisshin EM Co., Tokyo, Japan), stained with lead citrate and observed by HVEM. For radioautography, various organs of mice and rats were used after injection of radiolabeled compounds. To observe the intracellular localization of macromolecular synthesis, radiolabeled macromolecular precursors such as 3H-thymidine for DNA synthesis, 3H-uridine for RNA synthesis, 3H-amino acids for protein synthesis, 3H-glucosamine and 35SO4 for glucoide synthesis, 3H-fatty acids and 3H-glycerol for lipid synthesis were used (Nagata, 1998, 1999). Other radiolabeled compounds which bind to the specific cell organelles can also be used. We used 14C-bezafibrate, a peroxisome proliferator, which binds to endoplasmic reticulum and peroxisomes. The tissues were taken from the animals injected intraperitoneally with radio-labeled compounds 1 h before sacrifice and the tissues

Fig. 4. Medium magnification HV electron micrograph ((A), tilting +8°; and (B), tilting -8°) of a rat hepatocyte in primary culture, cultured in a normal Leibovitz L-15 medium for 3 days, prefixed in glutaraldehyde, stained with DAB reaction, postfixed in osmium tetroxide and dried by critical point drying. Many electron dense peroxisosmes (P) and less electron dense endoplasmic reticulum (E) surrounding the nucleus (N) can be seen. Accelerating voltage 1000 kV. The scale bar indicates 1 μm.
doubly fixed in 0.2% glutaraldehyde solution in cacodylate buffer (pH 7.4) and 1.0% osmium tetroxide solution in the same buffer, embedded in Epok 812 resin (Oken, Tokyo, Japan), sectioned at 0.2 μm thick, and processed for radioautography (Nagata, 1998).

2.2. Histochemical procedures

The various histochemical reactions employed were enzyme histochemistry, specific histochemical reactions and radioautography which were carried out as follows.

2.2.1. Enzyme histochemistry

Cultured cells were rinsed in Hanks’ solution (Nissui, Tokyo, Japan) for a few minutes, prefixed in 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer for 1 h, treated with the following incubating media, postfixed in 1% osmium tetroxide in the same buffer, rinsed in distilled water and finally dried by critical point drying.

2.2.1.1. Glucose-6-phosphatase for demonstration of endoplasmic reticulum. Cultured cells were incubated in a medium containing 0.125% glucose-6-phosphate and 2% lead nitrate for 30 min at 37°C, then they were substituted with cerium chloride (Wachstein and Meisel, 1956; Tice and Barrnett, 1962).

2.2.1.2. Thiamine pyrophosphatase for demonstration of Golgi apparatus. Cultured cells were incubated in a medium containing 2 mM thiamine pyrophosphate for 1 h at 37°C, then they were substituted with cerium chloride (Novikoff and Goldfischer, 1961).

2.2.1.3. Cytochrome oxidase for demonstration of mitochondria. Cultured cells were incubated in a medium containing cytochrome C (200 mg/dl) and DAB (100 mg/dl) for 1 h at 37°C, and were substituted with cerium chloride (Seligman et al., 1968).

2.2.1.4. Acid phosphatase for demonstration of lysosomes. Cultured cells were incubated in a medium containing 10 mM sodium glycerophosphate and 3 mM lead nitrate for 30 min at 37°C, and were substituted with cerium chloride (Mayahara and Chang, 1978).

2.2.1.5. DAB (di-amino-benzidine) reaction for demonstration of peroxisomes and pinocytotic vesicles. Cultured cells and embedded liver tissues were incubated in a DAB (3,3’-diamino-benzidine tetrahydrochloride, 10 mg/10 ml) solution for 10 min and postfixed in osmium tetroxide (Angermüller and Fahimi, 1981).

2.2.2. Specific histochemical reactions

2.2.2.1. ZIO (zinc iodide osmium) reaction for demonstration of Golgi apparatus. Cultured cells were stained in...
zinc iodide and osmium tetroxide solutions for 1 h (Vrensen and de Groot, 1974).

2.2.2. PA–TCH–SP (periodic acid–thiocarbohydrazide–silver proteinate) reaction for mucopolysaccharides in secretory granules. Semithin sections at 0.2 μm thick cut from the colons of ddY strain mice, fixed doubly in 2.5% glutaraldehyde and 1.0% osmium tetroxide in phosphate buffer at pH 7.4, were collected onto gold meshes (VECO, Eierbeck, The Netherlands) and stained by PA–TCH–SP procedures through 1% periodic acid solution, 0.2% thiocarbohydrazide in acetic acid solution and 1% silver proteinate solution (originally Thiéry, 1967 but slightly modified by Murata et al., 1977; Iida et al., 1978).

2.2.3. Radioautography

Radioautography was used for demonstrating the location of the radioisotope labeled compounds incorporated into cells and tissues after the administration either in vitro or in vivo. For the administration in vitro, HeLa S3 cells, CHO-K1 cells and primary cultured rat hepatocytes were plated in 35 mm plastic Petri dishes (Falcon Plastic, CA, USA) containing formval and collagen coated gold grid meshes (Nissin EM Co., Tokyo, Japan), cultured in a medium containing radioactive compounds, such as 3H-thymidine, 3H-uridine (Amersham, England, specific activity 877 GBq/mM, 37-1850 kBq/ml medium), 3H-amino acids, 3H-glucosamine and 35SO4, 3H-fatty acids and 3H-glycerol, which are precursors for macromolecular synthesis or 14C-bezafibrate (Boehringer Mannheim GmbH, Germany, specific
activity 2.83 MBq/mg, concentration 3.7 MBq/ml medium), which is a peroxisome proliferator and binds specific binding sites in cells, for times varying from 10 to 60 min. After the labeling, cells were fixed in 2% paraformaldehyde solution in phosphate buffer at pH 7.4 for 1 h, stained by DAB reaction, postfixed in 1% osmium tetroxide solution in the same buffer. The specimens were dehydrated in ascending ethanol series for a few minutes and dried in a critical point dryer.

For the administration in vivo, ddY strain mice at varying age were injected intraperitoneally with $^3$H-thymidine or $^3$H-uridine (Amersham, England, 37–1850 kBq/g.b.w.), 1 h before sacrifice. The liver and pancreatic tissues were taken out, fixed doubly in 2.5% glutaraldehyde solution in cacodylate buffer (pH 7.4) and 1% osmium tetroxide solution in the same buffer, dehydrated, embedded in Epok 812 resin (Oken, Tokyo, Japan), cut as 0.2 µm thick sections and picked up onto collodion coated copper EM grids. Both the whole mount cultured cells and thick sections were coated with Konica NR-H2 emulsion (Konica Co., Tokyo, Japan) according to a large wire-loop procedure (Nagata, 1998), exposed for 4–8 months and developed in SDX-1 developer or phenidone developer after GL latensification (Nagata, 1998), stopped and fixed in an acid fixer, stained with lead citrate and dried. By using thicker sections for radioautography, the exposure time can be reduced, since thicker sections contain more radioactivity than thinner sections.

3. High voltage electron microscopy and stereo viewing

All the specimens prepared as above were coated with a
thin layer of carbon and were observed in either JEOL JEM-4000EX intermediate high voltage electron microscope in our laboratory at 400 kV or Hitachi H-1250M ultra-high voltage electron microscope in the National Institute for Physiological Sciences, Okazaki, Japan, at 1000 kV. The EM grids were first observed without tilting (0° of tilt) and conventional photographs were taken (0°), then they were observed by tilting at +8° and −8°, and stereo-pair photographs were taken (+8° of tilt, and then −8° of tilt). The enlarged prints were observed with a stereoscope (stereoviewer, Peak No. 1994-4, Peak Ltd, Tokyo, Japan) to visually analyze the three-dimensional structures. Alternatively, a set of two lantern slides produced from the stereo-pairs were projected on a screen with two projectors through two polarizing filters crossing each other perpendicularly, either on vertical or horizontal lines, and were observed with a pair of polarizing glasses (commercially available, produced by Optical Co. such as Polaron, USA). Otherwise, the two pictures were looked at with the both eyes concomitantly, the right eye focusing on the left picture and the left eye focusing on the right picture separately and the two images were fused together in the optic center of the observer’s brain. When one becomes skilled in this procedure, the two pictures can easily be observed stereoscopically without any other facility. On the other hand, the two pictures of the stereo-pairs were further analyzed with an image analyzer JEOL JIM-5000 (JEOL, Tokyo, Japan), creating a stereo-color picture, with a red picture at the right side and a green (or cyan) picture at the left side composing into one, which was observed with the both eyes by using anaglyph type glasses (red/green or red/cyan glasses, JEOL, Tokyo, Japan), red glass for right eye and green (or cyan) glass for left eye. Alternatively, two contour lines plotted from the two pictures at a thickness or depth of 0.2 μm were composed into one and were observed with the anaglyph type glasses (JEOL, Tokyo, Japan) demonstrating the depth or heights of respective cell organelles similarly to the topographical geographic maps of mountains. Anaglyph type glasses are available at opticians or can be easily hand-made, by cutting glass frame from cardboard and pasting two sheets of red (right) and green or cyan (left) cellophane films onto the frame, respectively.

4. Three-dimensional observation of cell organelles

4.1. Enzyme histochemistry

4.1.1. Whole mount cultured cells

When whole mount cultured cells, either primary culture or established cell lines, which were stained with the various histochemical reactions and observed by HVEM at an accelerating voltage of 400–1000 kV, spread monolayered cells could be observed at low magnification, similar to light microscopy (Fig. 1). When cells were imaged at medium or high magnification (over 3000 ×), thin peripheral cytoplasm several micrometers thick could be easily imaged by 400 kV HVEM (Fig. 2). However, thicker perinuclear zones (over 10 μm) could not be imaged at 400 kV but only at

Fig. 8. Low magnification stereo-pairs of a hepatocyte of the liver of a rat fed with 2% DEHP containing diet for 2 weeks, prefixed in glutaraldehyde, stained with DAB reaction, postfixed in osmium tetroxide, embedded in Epoxy resin, sectioned at 0.5 μm, and observed by tilting. Many peroxisomes which are DAB positive appear electron dense and surround the nucleus at the center. (A) and (B): stereo-pairs ((A), tilting +8°, and (B), tilting −8°) should be observed with a stereoscope or by stereoscopic imaging. Accelerating voltage 1000 kV. The scale bar indicates 1 μm.
1000 kV. In all cases two photographs were taken in each field after tilting the specimen $+8^\circ$ and $-8^\circ$, then the enlarged stereo-pair prints were viewed with a stereoscope (stereo-viewer, Peak No.1994-4, Peak Ltd, Tokyo, Japan) or by stereoscopic imaging (Figs. 3 and 5). When anaglyph type color pictures are composed from the stereo-pairs using an image analyzer JEOL JIM-5000 (JEOL, Tokyo, Japan), the pictures should be observed with anaglyph type (red/green or red/cyan) glasses (Figs. 6 and 7). Thus the locations of cell organelles in cultured cells corresponding to the marker enzymes were three-dimensionally defined. The enzyme histochemical reactions observed were as follows.

4.1.1.1. Glucose-6-phosphatase. The G-6-Pase reaction, used to localize endoplasmic reticulum, showed three-dimensional structure of smooth surfaced endoplasmic reticulum stacked throughout the cytoplasm of cultured primary rat hepatocytes (Fig. 3A and B).

4.1.1.2. Thiamine pyrophosphatase. The thiamine pyrophosphatase (TPPase) reaction, which demonstrates electron dense positive end-products in the Golgi apparatus, showed networks surrounding the nuclei.

4.1.1.3. Cytochrome oxidase. The cytochrome oxidase reaction demonstrated many elongated spindle or spherical mitochondria disseminatedly in the whole cytoplasm of any cells. Many well developed filamentous mitochondria were seen in the cytoplasm of hepatocytes.

4.1.1.4. Acid phosphatase. The acid phosphatase (AcPase) activity demonstrated small spherical lysosomes in the cytoplasm of each cell type. In hepatocytes, many lysosomes were observed within the cytoplasm surrounding the nuclei.

4.1.1.5. DAB reaction. The established cell lines such as HeLa S3 or CHO-K1 cells did not show many DAB positive bodies in the cytoplasm, yet the primary cultured hepatocytes or subcultures showed several DAB positive peroxisomes alongside DAB negative endoplasmic reticulum (Fig. 4). However, when primary cultured hepatocytes were maintained in a medium containing...
clofibrate or bezafibrate, which are known to be peroxisome proliferators, many peroxisomes appeared in the cytoplasm (Fig. 5). Some hepatocytes showed electron translucent vacuoles enveloped with limiting membranes and including electron opaque DAB positive granules in the cytoplasm when they were cultured in a medium containing 0.2 mM clofibrate for 24 h, as can be observed with stereo–pairs (Fig. 5A and B). Such specific structures increased in number when the culture in clofibrate was extended for 48 h (Fig. 6). The three-dimensional structures and relative relations between respective granules can be well observed by means of stereo-pairs (Fig. 6A and B). Such specific structures increased in number when the culture in clofibrate was extended for 48 h (Fig. 6). The three-dimensional structures and relative relations between respective granules can be well observed by means of stereo-pairs (Fig. 6A and B). Such specific structures increased in number when the culture in clofibrate was extended for 48 h (Fig. 6). The three-dimensional structures and relative relations between respective granules can be well observed by means of stereo-pairs (Fig. 6A and B). Such specific structures increased in number when the culture in clofibrate was extended for 48 h (Fig. 6). The three-dimensional structures and relative relations between respective granules can be well observed by means of stereo-pairs (Fig. 6A and B). Such specific structures increased in number when the culture in clofibrate was extended for 48 h (Fig. 6). The three-dimensional structures and relative relations between respective granules can be well observed by means of stereo-pairs (Fig. 6A and B). Such specific structures increased in number when the culture in clofibrate was extended for 48 h (Fig. 6). The three-dimensional structures and relative relations between respective granules can be well observed by means of stereo-pairs (Fig. 6A and B). Such specific structures increased in number when the culture in clofibrate was extended for 48 h (Fig. 6). The three-dimensional structures and relative relations between respective granules can be well observed by means of stereo-pairs (Fig. 6A and B). Such specific structures increased in number when the culture in clofibrate was extended for 48 h (Fig. 6). The three-dimensional structures and relative relations between respective granules can be well observed by means of stereo-pairs (Fig. 6A and B). Such specific structures increased in number when the culture in clofibrate was extended for 48 h (Fig. 6). The three-dimensional structures and relative relations between respective granules can be well observed by means of stereo-pairs (Fig. 6A and B).
4.1.2. Thick sections

Semithin sections at 0.2 μm thickness stained for enzyme histochemistry can be observed by intermediate high voltage electron microscopy at accelerating voltages of 400 kV. However, thick sections at 0.5–2.0 μm cannot be easily observed at 400 kV, but require electron microscopy at 1000 kV (Figs. 8–10).

4.1.2.1. DAB reaction.

Rat liver tissues, fed with 2% DEHP (diethyl-hexyl phthalate) containing diet for 2 weeks were prefixed in 0.25% glutaraldehyde, incubated in a DAB medium, postfixed in 1% osmium tetroxide, embedded in Epok and sectioned at 0.5–2.0 μm were imaged, using stereo-pair pictures (Fig. 8A and B), which can be observed with a stereo-scope or by stereoscopic imaging.

Fig. 11. High power stereo-pair HV electron micrographs of a cultured rat hepatocyte, cultured in vitro for 3 days, fixed and stained with ZIO reaction. (A) and (B): stereo-pairs ((A), tilting +8°; and (B), tilting −8°) can be seen with a stereoscope or by stereoscopic imaging. Electron dense endproducts with ZIO reaction show networks of the Golgi apparatus surrounding the nucleus (N). Accelerating voltage 1000 kV (Copyright permission from Nagata, 1999). The scale bar indicates 1 μm.

Fig. 12. High magnification stereo-pair HV electron micrographs of a colonic goblet cell of a mouse intestine, fixed doubly in glutaraldehyde and osmium tetroxide in phosphate buffer and stained through periodic acid, thiocarbohydrazide and silver proteinate solutions (PA-TCH-SP). (A) and (B): stereo-pairs ((A), tilting +8°; and (B), tilting −8°) can be seen with a stereoscope or by stereoscopic imaging. Electron dense mucous granules piling up in the cytoplasm at center as well as in the goblet upper right can be seen. The scale bar indicates 1 μm.

4.1.2. DAB reaction. Rat liver tissues, fed with 2% DEHP (diethyl-hexyl phthalate) containing diet for 2 weeks were prefixed in 0.25% glutaraldehyde, incubated in a DAB medium, postfixed in 1% osmium tetroxide, embedded in Epok and sectioned at 0.5–2.0 μm were imaged, using stereo-pair pictures (Fig. 8A and B), which can be observed with a stereo-scope or by stereoscopic imaging.
In the cytoplasm of these hepatocytes many electron dense peroxisomes can be seen three-dimensionally, surrounding the nucleus (Fig. 8). When such cytoplasm is observed by high magnification, DAB positive irregular shaped peroxisomes such as tadpole shape or disk-like shape (Fig. 9) can be observed three-dimensionally when viewed by HVEM stereo-pairs. The liver tissues obtained from rats fed with 2% DEHP containing diet for 1 week showed fewer peroxisomes than the tissues fed for 2 weeks. The ultrastructure and morphological relations between peroxisomes and other cell organelles such as endoplasmic reticulum or mitochondria can be seen using stereo-pair pictures (Fig. 10A and B), or composed color-pictures from the stereo-pairs (Fig. 10C), composed color pictures from contour lines and original picture (Fig. 10D) or two contour lines (Fig. 10E) can be seen with anaglyph type glasses. Thus, the height of each structure sectioned at 0.2 μm can be measured and the contour lines drawn with an image analyzer (JEOL JIM-5000) to demonstrate the height or depth of each structure.

4.2. Specific histochemical reactions

4.2.1. Whole mount cultured cells

4.2.1.1. ZIO reaction. The ZIO reaction produced high electron dense endproducts over the Golgi apparatus of all cell types. In cultured hepatocytes, for example, the contours of Golgi apparatus showed electron dense networks in the perinuclear region of the hepatocyte cytoplasm surrounding the nucleus, which can be seen with the stereo-pair pictures (Fig. 11A and B).

4.2.2. Thick sections

4.2.2.1. PA–TCH–SP reaction. The PA–TCH–SP reaction for mucopolysaccharides was applied to the colonic epithelial cells of normal ddY mice. The positive reaction with electron dense deposits appeared in the mucous granules in the Golgi area as well as the goblets in the apical cytoplasm of the goblet cells in the colonic epithelium. The mucous granules in the Golgi area and the goblets can be observed three-dimensionally as superimposed particles in stereo-pairs (Fig. 12A and B).

4.3. Radioautography

4.3.1. Whole mount cultured cells

4.3.1.1. Macromolecular precursors. Radioautograms prepared from cultured cells labeled with macromolecular synthesis in vitro such as ^3^H-thymidine incorporated into
DNA or \(^3\)H-uridine into RNA can be observed by HVEM at 400–1000 kV. It was revealed that silver grains due to \(^3\)H-thymidine and \(^3\)H-uridine were localized over the nuclei of cultured cells.

4.3.1.2. Specific binding sites. To demonstrate the specific binding sites of peroxisome proliferators, rat hepatocytes in primary culture were labeled in vitro with \(^14\)C-bezafibrate (Boehringer Mannheim GmbH, Germany, specific activity 2.83 MBq/mg, concentration 3.7 MBq/ml medium) for 10–60 min, then fixed and radioautographed. The silver grains due to \(^14\)C-bezafibrate were observed over the cytoplasm of all the cultured hepatocytes. The grains were localized over the endoplasmic reticulum and peroxisomes of the cultured hepatocyte cytoplasm. In the stereo-pairs (Fig. 13A and B), grains were concentrated over the endoplasmic reticulum as well as over the peroxisomes, demonstrating three-dimensionally the specific binding sites of this radioactive compound.

4.3.2. Thick sections

4.3.2.1. Macromolecular precursors. Radioautograms prepared from semithin (0.2 \(\mu\)m) to thick (0.5 \(\mu\)m) sections of the liver and pancreatic tissues incorporating \(^3\)H-thymidine (Fig. 14) and \(^3\)H-uridine (Fig. 15) can be readily observed by HVEM at 400–1000 kV, with localization of silver grains over the nuclear chromatin. By using thicker sections, the exposure time can be reduced because these contain more radioactivity than thinner sections (Nagata, 1995, 1996, 1998). The grains due to \(^3\)H-amino acids such as \(^3\)H-leucine or \(^3\)H-proline are localized over endoplasmic reticulum, while \(^3\)H-glucosamine are over the Golgi apparatus in the hepatocytes, demonstrating the synthetic sites of these macromolecules (Nagata, 1996, 1997b). When thicker sections, as thick as 0.2 \(\mu\)m, were observed by conventional electron microscopy at accelerating voltages of 100 kV, the ultrastructure of the cell organelles and the silver grains were not clearly visible and we could not discriminate the
electron density between the cell organelles and the silver grains (Fig. 15A). However, when we observed the same specimens by HVEM at 400–1000 kV, the silver grains appear much denser than the cell organelles and the silver grains can be well differentiated from the cell organelles (Fig. 15B). By using stereo-pairs, the three-dimensional relationship between the cell organelles and the silver grains can be well defined.

5. Discussion

From the results obtained using both cultured cells and thick sections, various subcellular organelles such as Golgi apparatus, endoplasmic reticulum, mitochondria, lysosomes, peroxisomes, pinocytotic vesicles, secretory granules can be defined by means of marker enzymes or specific histochemical reactions as G-6-Pase, TTPase, cytochrome oxidase, AcPase, DAB, ZIO, PA–TCH–SP reactions or radioautographic silver grains showing localization of macromolecular synthesis such as DNA, RNA, proteins, glucides, lipids and specific binding sites three-dimensionally by HVEM and stereo-viewing. These histochemical reactions can be localized three-dimensionally in thick specimens such as whole mount cultured cells or thick sections from embedded tissues. This procedure should be very useful in localizing and assessing the structures of these cell organelles, as well as for the analysis of their three-dimensional inter-relationships.

Observation of whole mount cultured cells by electron microscopy was first carried out by Porter et al. (1945) who tried to observe thin cytoplasmic processes without sectioning at the initial stage of conventional electron microscopy. Then, they later tried again to observe the cytoskeletons of whole mount cultured cells by HVEM at the accelerating voltage of 1000 kV, without any histochemical reactions after drying with a critical point dryer (Buckley and Porter, 1975; Wolosewick and Porter, 1975, 1976, 1977, 1979).

We have used a similar technique to observe cultured cells by HVEM at 400–1000 kV during the 1980s and 1990s using enzyme histochemical reactions by identifying the marker enzymes such as G-6-Pase, TTPase, cytochrome oxidase, AcPase, DAB, by specific histochemical reactions such as ZIO, PA–TCH–SP and radioautography by incorporation of radioactive macromolecular precursors or specific binding compounds (Nagata et al., 1977, 1986, 1987, 1990; Nagata and Usuda, 1984; Nagata, 1995, 1998, 1999; Momose and Nagata, 1993). Biological materials other than cultured cells were also used to study the ultrastructure of single cells and microorganisms. Isolated HTLV-1 particles associated with myelopathy patients were observed using HVEM by Owaki (1989), after staining with lectins. Several strains of whole mount cultured cells obtained from mammals were observed after staining with histochemical reactions. The cytoskeletons in cultured epithelial cells and glioma cells were demonstrated immunohistochemically (Sasaki et al., 1991, 1996; Taomoto, 1996). The mesangial...
cells and endothelial cells obtained from the aortae and kidneys of adult rats were cultured in vitro and stained immunohistochemically (Oite et al., 1995, 1996). Three-dimensional ultrastructures of cells and tissues obtained from several organs of mammalian and lower vertebrate animals, fixed, embedded and thick sectioned were recently observed by three-dimensional HVEM. Sarcoplasmic reticulum in the skeletal muscle fibers of rat and mice stained by silver impregnation was observed in thicker sections by Hayashi and Nagata (1991). Nematolysosomes in the liver, pancreas, kidney and salivary gland of rats and mice stained with acid phosphatase reaction was demonstrated by Sakai et al. (1992). The three-dimensional structure of the processes of neurons and neuroglial cells were also studied using thicker sections by HVEM such as neurons and neuroglia in the rat brains (Hama, 1989; Hama et al., 1994), synaptic junctions in the rat cereberum (Igarashi et al., 1988), retinal neuronal cells in the fish eyes (Hidaka et al., 1993; Hidaka and Hashimoto, 1995). The period from the 1980s to the 1990s should be regarded as the third period of using HVEM to observe cell organelles in various cells in combination with histochemical reactions.

Since the recent development of intermediate high voltage electron microscopes around 300–600 kV, which are less expensive than the very high voltage electron microscopes at 1000 kV and now available in conventional laboratories (as well as access to very high voltage electron microscopes at 1000 kV or more), it has become easy to make use of such instruments for analyzing the three-dimensional structures and distribution of cell organelles in single cells and tissues.

In order to demonstrate specific cell organelles, specific marker enzymes can be used, such as G-6-Pase for endoplasmic reticulum, TPPase for Golgi apparatus, cytochrome oxidase for mitochondria, acid phosphatase for lysosomes, and DAB for peroxisomes. Some other histochemical reactions specific for individual cell organelles are also appropriate, such as ZIO for Golgi apparatus, PA–TCH–SP for mucous goblets, lectins for cell surface membranes, immunohistochemistry for cytoskeletons, and silver impregnation for myofibrils and neurofibrils, and radioautography for DNA, RNA, proteins, glucides, lipids and specific binding sites.

6. Concluding remarks

Thick biological specimens, both whole mount cultured cells and thick sections cut from embedded tissue blocks, stained with various histochemical reactions can be easily observed by HVEM to demonstrate the location of cell organelles corresponding to the specific histochemical reactions. We have developed the necessary methods to prepare thick specimens from both whole mount cultured cells on grids or semithin and thick sections from epoxy resin embedded tissues and to stain them with various histochemical reactions to localize cell organelles for observation by intermediate or HVEM at accelerating voltages (400–1000 kV) and to analyze the intracellular localization and ultrastructure of cell organelles with the stereo-pair images. This procedure is helpful for the understanding of the three-dimensional structure of cell organelles and their ultrastructural relationships in different cells and tissues. Future applications of this approach can be anticipated.

Acknowledgements

The author wishes to express his sincere gratitude to Drs Kiyoshi Hama and Tatsu Arii, National Institute for Physiological Sciences in Okazaki, for providing the facilities to use the Hitachi H-1250 high voltage electron microscope in their Institute, and to Mr Kiyokazu Kametani, Electron Microscopy Laboratory, Shinshu University School of Medicine, Matsumoto, for his technical assistance during the course of this study. This study was supported in part by a Grant-in-Aids for Scientific Research from the Ministry of Education, Science and Culture of Japan, as well as a Grant for Promotion of Characteristic Research and Education from the Japan Foundation for Promotion of Private Schools.

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