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Repeated Note on the Significance of Embedment-Free Section Transmission Electron Microscopy. A Review

Nota Repetida sobre la Importancia de la Microscopía Electrónica de Transmisión de Secciones Libres de Incrustación. Una Revisión

Surang Chomphoo[1]; Wiphawi Hipkaeo[1] & Hisatake Kondo[1,2]

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SUMMARY: After a concise review of the history of why the embedment-free section transmission electron microscopy (TEM) was developed, two major characteristics/advantages of this method were described: 1) enhanced electron-translucency of bio-specimens, and 2) disclosure of strand networks occupying the cytoplasmic matrix. The former advantage, the main issue of this review, makes it possible to see-through superimposed laminated targets when viewed en-face. Ultrastructural features of the basement membranes in the renal glomerular filtration barrier of rats and in the synapse of Torpedo electrocytes, and those of thinner myelin sheaths were noted as 3D examples of this electron-translucency. Such seeing-through of the basement membranes disclosed regional structural heterogeneity suggesting their remodeling and dynamics. It also has the potential to analyze the spatial interrelation of bioactive molecules localized in the superimposed compartments partitioned by the laminated targets. As for the latter advantage, though avoiding its details in this review, the possibility has repeatedly been proposed that the cytoplasmic networks as a whole, but not individual strands themselves, represent the concentration of cytoplasmic matrix proteins and their sol or gel status. The possible interpretation of the cytoplasmic networks as representing the cytoplasmic sol-to-gel transition may shed new light on understanding the mechanisms of intracellular dynamics discrete from the known idea on the cytoskeleton. With its two characteristics/advantages in consideration, the embedment-free section TEM is still worth attracting more attention in ultrastructural analyses of bio-specimens.

KEY WORDS: Embedment-free section; Electron-translucency; en-face basement membranes; Cytoplasmic strand networks; Cytoplasmic sol-to-gel.

INTRODUCTION

The reason why such cytoplasmic strand networks in the embedment-free section TEM (transmission electron microscopy) were disclosed was interpreted as follows: The cytoplasmic strand networks have electron-scattering properties (representing the contrasts in TEM) similar to that of the epoxy embedding media and they consequently appear vague or indistinct in conventional epoxy-embedded section TEM. In contrast, owing to the absence of epoxy embedding media in the embedment-free section TEM, the strand networks appear in enhanced contrast (Wolosewick & Porter, 1979). In brief, a key issue is how much the epoxy embedding media interferes with the contrast/visibility as well as the electron-translucency of any cellular structural components. This interference by the epoxy embedding media is further confirmed when freezing sections of chemically fixed specimens at the thickness close to 1 mm on a cryostat, though not easy to make actually, are observed in TEM under the regular accelerating voltage after the process with CPD. With this process of specimen preparation, we can recognize clear visibility of various subcellular structures (please refer to Figure 1 in Kondo (2006), in contrast to our usual experience that such thick sections, if embedded in epoxy resin, appear highly dark and could not show any intracellular details in the regular TEM.

On the basis of the interpretation, subsequent to the HTEM (high voltage TEM) study of whole-mounted cells, another embedment-free TEM method was developed, in

[1]Electron Microscopy Unit, Department of Anatomy, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.
[2]Department of Anatomy, Graduate School of Medicine, Tohoku University, Sendai, Japan.

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which polyethylene glycol-embedding and eventual de-embedding after sectioning were employed (Wolesewick, 1980). As a result, any *in situ* tissue cells were able to be observed in embedment-free sections under a regular accelerating voltage of 100 kV or less. This transiently PEG-embedded and eventually embedment-free section TEM method in comparison with the conventional epoxy-embedded section TEM method is schematically shown in Figure 1. With the then novel method, Wolosewick (1980) disclosed that the cytoplasm of several *in situ* cells appeared with high contrast without any TEM staining and strikingly resembled that of the whole-mount unembedded cultured cells in HTEM. Following the development by Wolosewick (1980), Kondo, one of the present authors, performed a series of studies analyzing various *in situ* cells utilizing the embedment-free section TEM method and disclosed ultrastructural features of any *in situ* cells quite similar to those in the HTEM including the cytoplasmic strand networks (Kondo *et al*., 1980, 1982; Kondo, 1984a,b). In addition, the histological architecture of tissues and cells in the embedment-free section TEM was shown not to be dissimilar to those in conventional epoxy-section TEM (Kondo, 2006, 2008).

It is consequently evident that the embedment-free section TEM presents two characteristic features in ultrastructure whose significance should be considered: the enhanced electron penetration into bio-specimens and resulting translucency of several bio-structures under a regular accelerating voltage of 100 kV or less, and the disclosure of strand networks in the cytoplasmic matrix. The nature of the cytoplasmic strand networks has been reviewed several times and their possible significance as the phase transition of sol and gel of the cytoplasmic matrix has already been proposed (Kondo, 2010; Hipkaeo & Kondo, 2016). Therefore, the main attention in the present review was focused on the enhanced electron penetration and resulting translucency of several bio-structures.

What are disclosed from the enhanced electron-translucency in the embedment-free section TEM?.

Please notice again the fact that not only the cytoplasmic strand networks but also all

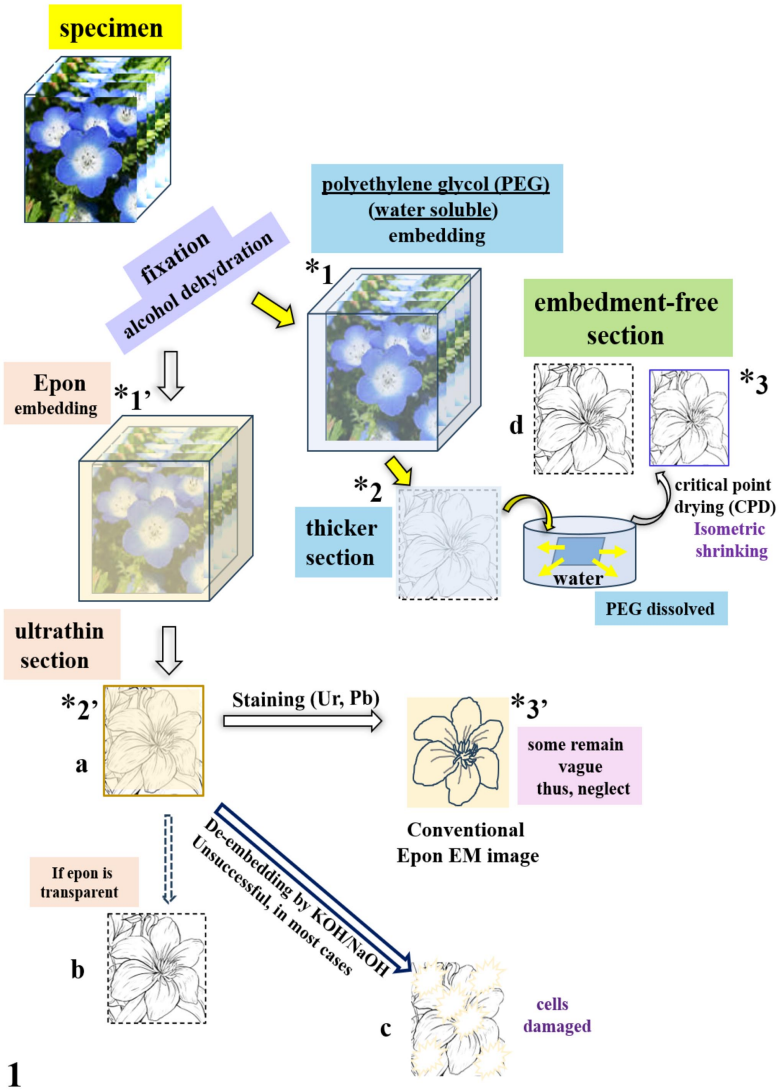

Fig. 1. Schematic drawing of flow charts about embedment-free section TEM method (yellow arrows) in comparison with conventional epoxy TEM (white arrows). Differences between the former and the latter are: embedding in PEG (*1) versus Epon (*1'), thicker section (*2) *versus* ultrathin section (*2'), embedment-free section (*3) by water immersion with CPD-induced isometric shrinkage (in comparison with supposed one (d)) and without staining *versus* Epon-embedded (a) & stained section (*3'). Epoxy embedment-free trial with KOH or NaOH, which has theoretically epon-solving effects, results in serious damage to specimens (c) and miserable ultrastructural images.

known organelles were clearly recognized in whole-mounted culture cells in HTEM as well as the early-day TEM (Porter *et al*., 1945; Wolosewick & Porter, 1979). That finding indicates the plasma membranes themselves to be electron-translucent when seen *en-face*. In contrast, a question whether or not the plasma membranes as well as intracellular membranes are electron-translucent in their *en face* view has probably not been under consciousness to most TEM observers, because the bio-membranes as electron-dense lines in their cut view right to their planes in TEM have been strongly impressed to most observers.

This unexpected translucency is also the case in the basement membranes whose common image comes from their cut-view observation as a substantial width of high electron density, resulting in its rather common imagination as electron-opaque even when viewed *en face*. However, using the embedment-free section TEM, the electron-translucency to a considerable extent, but not the electron-opacity, of the basement membranes, when observed in *en face*, has been demonstrated in tangentially-cut embedment-free sections of the renal glomerular filtration barrier of rats (Kondo, 1990, 2011; Kondo & Hipkaeo, 2013). This feature is summarized in a schematic drawing in comparison with that in the conventional epoxy-section TEM (Figs. 2a-d). In the renal glomerulus, the urine filtration barrier is composed of three layers: the fenestrated endothelium, the basal lamina, and the pedicles of the epithelial podocyte. In its tangential sections, one, two, or all of the three layers must be located in singular or multiple-superimposed ways at sites near the margin of the sectioned filtration barrier. In conventional epoxy TEM, however, each of the three layers is recognized only when its portions are located at the top surface of the sections owing to the presence of epon interfering with sufficient electron-penetration (Figs. 2b,c). In contrast, in the embedment-free section TEM, it is possible to see *en face* basement membranes through the slit spaces between adjacent pedicles of the podocytes at sites of the double superimposition (Fig. 2d), or through both endothelial fenestrae and slit spaces at sites of the triple superimposition.

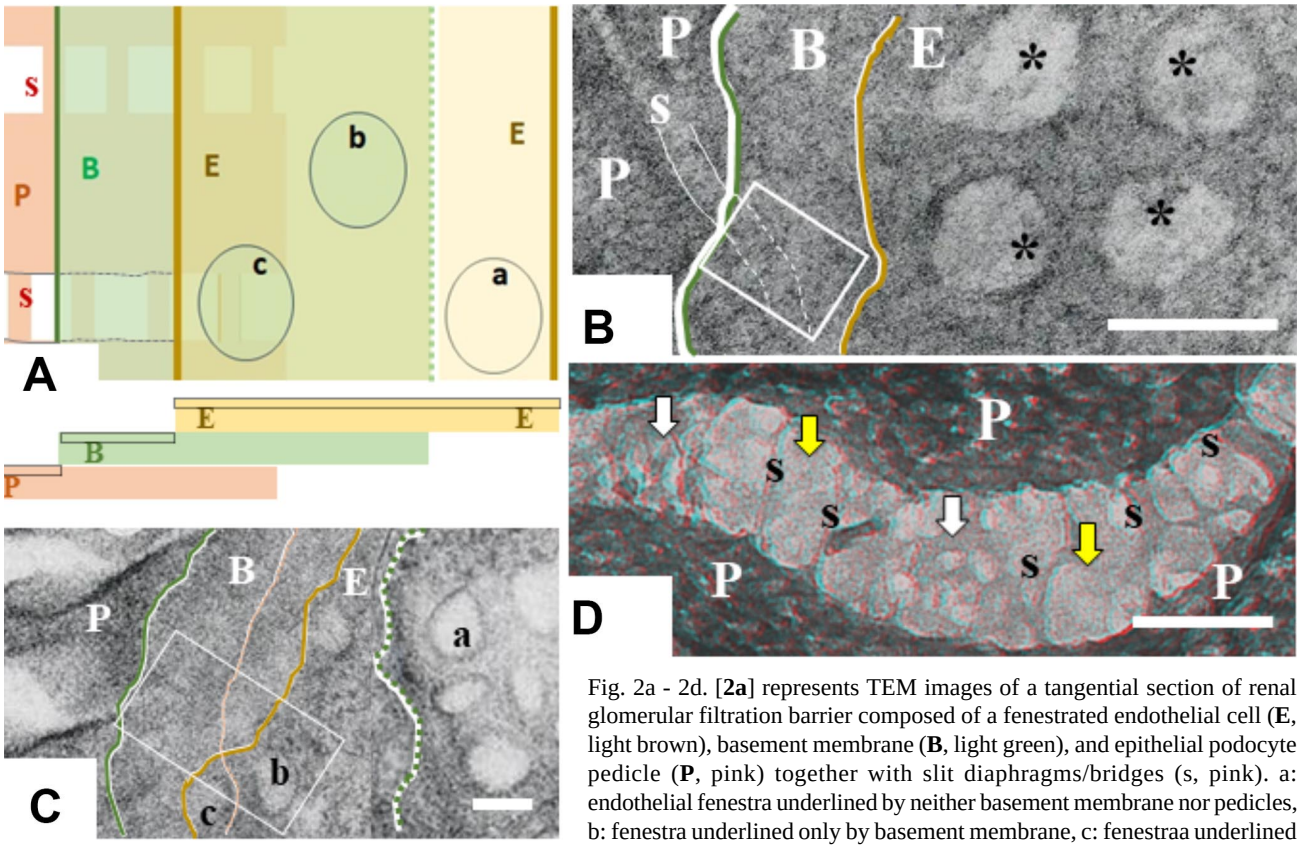

Fig. 2a - 2d. [**2a**] represents TEM images of a tangential section of renal glomerular filtration barrier composed of a fenestrated endothelial cell (**E**, light brown), basement membrane (**B**, light green), and epithelial podocyte pedicle (**P**, pink) together with slit diaphragms/bridges (s, pink). a: endothelial fenestra underlined by neither basement membrane nor pedicles, b: fenestra underlined only by basement membrane, c: fenestraa underlined by both basement membrane and pedicles. Thin rectangles with dotted lines represent areas visible *en face* in conventional TEM in contrast to three entire laminae visible in the embedment-free section TEM. [**2b**] and [**2c**] represent conventional TEMs of tangential sections of the filtration barrier at lower and higher magnifications., respectively. In [**2b**], the supposed cutting edges of a fenestrated endothelium (E) and basal lamina (B) are shown in lines of brown, and green, respectively. Broken lines of green and pink represent corresponding edges covered by the superimposed components. In [**2c**], a higher magnification view of an area corresponding to that marked by a white rectangle in [**2b**] is shown. Green and brown lines indicate the cutting edges of basal lamina (B) and endothelium (E), respectively. Lumens (*) of endothelial fenestrae appear vague, indicating that the underlined basement membrane is not structurally recognized. An area marked by a white rectangle, although it appears homogeneous without any distinct structural components, is considered to be formed by superimposition of basement membrane (B) and pedicles (P) whose contours are indicated by thin white lines (their dotted lines represent supposed contours overlapped by basement membrane). In [**2d**], an area corresponding to the white rectangle-marked area in [**2c**] is shown in the embedment-free section TEM. Note *en face* views of the basement membrane as a lamella of lower electron density with several slit diaphragms/bridges (s) superimposed between two adjacent pedicles (P). Also note holes in the basement membrane and regional differences in the number and sizes of the holes: domains marked by white arrows have groups of small holes and domains marked by yellow arrows have relatively homogenous matrix. Bars represent 100 nm.

At the location of multiple superimpositions, when viewed in 3D, two or three of such components as pedicle including the slit bridges/diaphragms, *en face* basement membranes, and endothelial fenestrae are individually differentiated. For details of findings in this regard, especially 3D TEM images, please refer to Figures 6 and 7 in Kondo (1990) observable with the naked eye, and Figure 8 in Kondo (2011) with the red and blue glass. In brief, the basement membrane is sufficiently translucent to electrons. Although the basement membrane appears as an evenly expanded film as already expected from its conventional epoxy TEM image, it should be noted that irregular holes are contained in the film, and the hole sizes and numbers varied at different domains of the film (Fig. 2d). The regional heterogeneity in the appearance of such holes may represent a dynamic change of the basement membrane, resulting in the regional heterogeneity of the urine-filtering function, which remains to be elucidated.

Another case of the electron-translucency of the basement membrane has recently been published in a study by the present authors, in which the synaptic basement membrane from *Torpedo* ray electrocytes was analyzed (Chomphoo *et al.*, 2024). In tangential sections of the electrocyte synapse, synaptic vesicles in the presynaptic terminal were seen through the synaptic basement membrane when viewed from the postsynaptic electrocyte characterized by fibrous components, or vice versa, that is, fibrous components in postsynaptic electrocytes seen through the synaptic basement membrane from presynaptic terminals. This clearly indicates the electron-translucency of the basement membrane (Figs. 3a-c). In addition, the *en face* view in the embedment-free section TEM disclosed for the first time the presence of focal defects of irregular shapes in the synaptic basement membrane, which corresponded to interruptions of the dense line representing the cut-view of the synaptic

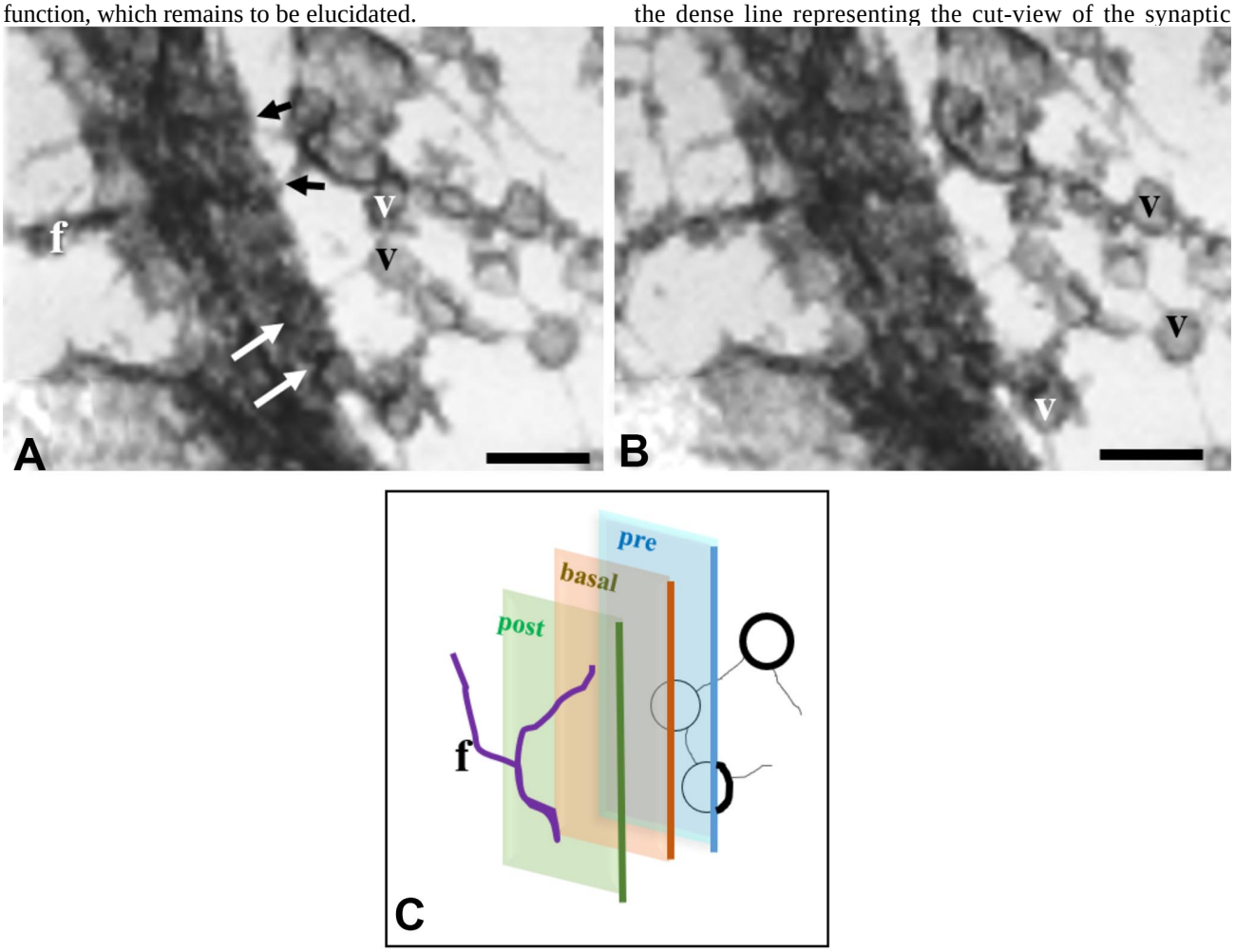

Fig. 3a -3c. [3a] and [3b] represent two embedment-free electron micrographs as a stereo pair of a section of Torpedo synapse with tilting at 7° each other. A plane of the synapse is tilted about 75° to the section plane. A 3D image is appreciated with both naked eyes briefly trained for stereo-viewing. Note some (white arrows) of synaptic vesicles (v) close to the presynaptic membrane are visible through the almost *en face* basement membrane plus synaptic plasma membranes. Parts of cytoplasmic strands (black arrows) in association with synaptic vesicles are continuously visible through the basement membrane. [3c] represents a schematic drawing of architectural 3D synapse constructed from a pair of [3a] and [3b]. Purple lines represent fibrous elements (f) in the electrocyte. Bar represents 100 nm.

basement membrane in the conventional TEM. Such regional heterogeneity in the synaptic basement membrane, detectable for the first time by *en face* TEM viewing of the synapse structure, may represent a dynamic change of the basement membrane, resulting in the regional heterogeneity of the synaptic functions.

In addition, the neural myelin sheath, unless it is too thick, has also been shown to be electron-translucent in 3D in the embedment-free section TEM, and intra-axonal cytoskeletons and extra-axonal/interstitial structural entities were viewed through the myelin (please refer to Fig. 2 in Kondo, 2006). This feature is understandable considering that the myelin is composed of superimposed wrapping of the plasma membrane which is electron-translucent as noted in the previous section.

Perspective.

The embedment-free section TEM attracts much less attention at present than almost half a century ago when it was developed following the idea of the structured cytoplasm advocated by Porter (Wolosewick & Porter, 1979; Porter, 1984, 1987). However, in view of the two major characteristics of this method as described above: 1) the electron-translucency of specimens and 2) the disclosure of the strand networks occupying the cytoplasmic matrix of almost all cells, this method is still worth attracting more attention in ultrastructural analyses of cells and tissues.

The first characteristic, the main issue of this review, makes it possible to see-through laminated targets when viewed *en face*, as exemplified in the renal glomerular filtration barrier, and the synapse in this article. It is natural from the scientific viewpoint to have a desire of seeing-through any biological cells and tissues under light in the naked eye and LM, although most biological targets are light-opaque because their surface layers and interiors are rich in molecules absorbing or reflecting light (Cruz & White, 2022). The same must be the case in TEM. In this regard, it should be noted that the basement membrane functionally interacts with the plasma membranes of cells facing to it and that the plasma membranes further interact with organelles of their enveloping cells in the glomerular urine filtration barrier, the neuro-muscular junction (NMJ) and its derivative representing the electrocyte synapse (Köhling *et al*., 2006; Rastaldi *et al*., 2006; Pozzi *et al*., 2017). It is also known that, in development and maintenance of the myelin, the interaction between protein molecules in the extracellular matrix proteins and the myelin-generating cell membranes plays important roles, as exemplified by collagen III and IV and laminin-211 in the extracellular matrix versus GPR56 and 126 of adhesion G protein-coupled receptors (aGPCRs) in the membranes (Mehta & Piao, 2017). Therefore, the seeing-through of superimposed components in the urine filtration barrier and the NMJ-derived electrocyte synapse as well as the neural myelin by the embedment-free section TEM has potential and advantage to analyze spatial interrelations of bioactive molecules localized in the superimposed compartments when the immuno-gold labeling is applied using specific antibodies to them. Because specimens are embedment-free and naked to any antibodies applied to the sections for the immuno-reaction, the immuno-gold labeling in TEM has already been shown to be highly efficient and distinct by means of this method (please refer to Fig. 18 in Kondo & Ushiki, 1985).

As for the second characteristic, details of its significance should be referred to several previous literatures (Kondo, 2010; Hipkaeo & Kondo, 2016). Briefly saying, from demonstration of different extents of the compactness of networks of a protein gelatin at cool and warm temperatures with this method, a possible interpretation was led that the strand networks, but not their strands themselves, represent the sol/gel transition of the cytoplasmic matrix. The possible interpretation was supported by different extents of the compactness of the cytoplasmic networks in the cell cortex (loose ones) and the deep interior (compact ones) of amoeba cytoplasm (Kondo, 1995), whose compactness feature is compatible with the established idea on the mechanism of the amoeba movement (Allen, 1973). Any further physicochemical examination related to the sol/gel phase transition in combination with this method is necessary for further reinforcement of the idea. This possible interpretation that the cytoplasmic networks 'as a whole' represent the cytoplasmic sol/gel transition, although not compatible with the presently dominant idea based on understanding 'one molecule to one structural element' in the cytoskeleton, may shed new light on understanding the intracellular dynamics discrete from the known viewpoint based on the cytoskeleton.

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RESUMEN: Después de una revisión concisa de la historia del motivo del desarrollo de la microscopía electrónica de transmisión (TEM) de sección libre de incrustación, se describieron dos características/ventajas principales de este método: 1) translucidez electrónica mejorada de las muestras biológicas, y 2)

divulgación de Redes de hebras que ocupan la matriz citoplasmática. La primera ventaja, el tema principal de esta revisión, hace posible ver a través de objetivos laminados superpuestos cuando se ven de frente. Las características ultraestructurales de las membranas basales en la barrera de filtración glomerular renal de ratas y en la sinapsis de los electrocitos Torpedo, y las de las vainas de mielina más delgadas, se observaron como ejemplos tridimensionales de esta translucidez electrónica. Tal visión a través de las membranas basales reveló heterogeneidad estructural regional, lo que sugiere su remodelación y dinámica. También tiene el potencial de analizar la interrelación espacial de moléculas bioactivas localizadas en los compartimentos superpuestos divididos por los objetivos laminados. En cuanto a esta última ventaja, aunque evitando sus detalles en esta revisión, se ha propuesto repetidamente la posibilidad de que las redes citoplasmáticas en su conjunto, pero no las hebras individuales en sí, representen la concentración de proteínas de la matriz citoplasmática y su estado de sol o gel. La posible interpretación de las redes citoplasmáticas como representación de la transición citoplasmática de sol a gel puede arrojar nueva luz sobre la comprensión de los mecanismos de la dinámica intracelular distintos de la idea conocida sobre el citoesqueleto. Teniendo en cuenta sus dos características/ventajas, aun vale la pena atraer más atención a la sección TEM sin incrustación en los análisis ultraestructurales de muestras biológicas.

PALABRAS CLAVE: Sección libre de empotramiento; Translucidez electrónica; membranas basales enfrentadas; Redes de hebras citoplásmicas; Sol-gel citoplasmático.

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Corresponding author:
Surang Chomphoo
Electron Microscopy Unit
Department of Anatomy
Faculty of Medicine
Khon Kaen University
Khon Kaen
40002
THAILAND

E-mail: suranch@kku.ac.th

https://orcid.org/0000-0003-2879-5127