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Lessons from Literature of Prominent Pioneers in the Early Days of Electron Microscopic Analysis of Cells (1940s-1950s)

Lecciones de la Literatura de Destacados Pioneros en los Primeros Días del Análisis Microscópico Electrónico de Células (1940s-1950s)

Hisatake Kondo & Wiphawi Hipkaeo

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SUMMARY: An overview is given of the history of transmission electron microscopy (TEM) with a focus on several questions: what were the major ways in which TEM enhanced our knowledge of the cell structure beyond that gained in light microscopy (LM); how were intracellular structures not recognized in LM newly disclosed in TEM; how was the reality of newly observed intracellular structures established, and how important was baseline information accumulated during the long LM history to priming scientists using TEM for the task of finding new intracellular structures. It is hoped that this review will encourage young researchers to discover details of the morphology of cells and tissues in the near future.

KEY WORDS: Electron microscopy; Early days of bio-EM; Hyaloplasm; Light microscopy; Reality or artifacts.

INTRODUCTION

In any field of science, a drive to look at finer and finer details of the world around us has been a major driving force in their early development. This was especially the case in histology. Antony van Leeuwenhoek in Holland made the earliest optical magnifying device, a light microscope. Utilizing light microscopy (LM), by the mid-20th century, the concept of cell structure had been established as follows: the cell, including the nucleus, was composed of protoplasm. The nuclear protoplasm was termed nucleoplasm, the remainder was termed cytoplasm. The cytoplasm was divided into morphoplasm and hyaloplasm. The morphoplasm represented varieties of tiny entities that were visible in LM as dots or lines, while the hyaloplasm represented domains that appeared homogenous and unstructured. As components of the morphoplasm, LM histology had already recognized mitochondria, Golgi bodies, centrosomes, vacuoles, glycogen, lipid droplets, tonofibrils, myofibrils, and cilia. How the LM-derived knowledge of cells was enhanced by the introduction of transmission electron microscopy (TEM) to the life sciences is the main topic of this article.

Initial struggles in the application of TEM to life sciences.

TEM itself was invented/developed in the physics field in the 1930s. In the Technische Hochschule Berlin, under Max Knoll, Ernst Ruska succeeded in the construction of an electron microscope with a magnification of x16, and then of another microscope with three magnetic lenses, functioning condenser, objective, and projector, and having a magnification of x12,000. For his contributions, Ruska was a Nobel laureate in 1986 (Ruska, 1986). Following his success, TEM was pursued in several countries such as Belgium, Britain, France, Japan, and the USA. During and after World War II, commercial TEMs were developed by several electronics companies such as Siemens, RCA, Philips, Hitachi, Jeol, Akashi, and Shimazu (Haguenau *et al*. 2003). Early in the development, attempts to study cell structure were made in various laboratories. The resulting image quality, however, was generally disappointing during the period covering the late 1930s and early 1940s. Subsequently, a substantial number of bio-scientists with long experience in histology started to be involved in the development of bio-TEM, resulting in progressive advancement in methodology and understanding of cell substructures.

Electron Microscopy Unit, Division of Anatomy, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.
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Prominent among them were the USA Rockefeller Institute group including August Claude, Keith R Porter, and George E Palade, as well as Sweden's Karolinska Institute group headed by Fritiof Sjöstrand. A high level of competition among them is easily imagined from their articles: they often added notes in their full papers stating that the findings had been presented as proceedings in previous scientific meetings. Claude and Palade among others were Nobel laureates (together with de Duve) in 1974 for their discoveries concerning the structural and functional organization of the cell. Concise summaries on the history of TEM itself and bio-medical TEM by Claude (1947, 1975), Palade (1971), and Haguenau *et al*. (2003) are informative.

Three major contributions of TEM to our understanding of cell structure can be identified. First and foremost was the discovery of the membrane at the cell surface, which confirmed the distinct and real existence of the cell membrane, a structure suspected to exist on the basis of LM. The second contribution was the disclosure that membranes delineated many intracellular components, some of which were already known to exist in the morphoplasm such as mitochondria, Golgi bodies, and secretory granules as well as cell nuclei. The third was the new identification of structural components in the hyaloplasm, such as the endoplasmic reticulum which is invisible in LM. In addition, non-membranous ribosomes and cytoskeletal components were identified in the hyaloplasm. Parts of the hyaloplasm lacking structures in TEM even after new identification of various organelles and inclusions are now termed the cytoplasmic matrix/ ground substance. In other words, the cytoplasmic matrix is defined as intracellular domains remaining after the conceptual exclusion of all cell organelles and inclusions including the cytoskeleton. In more recent times, there has been a series of ultrastructural studies by Porter (Wolosewick & Porter, 1979) to clarify the nature of the cytoplasmic matrix in high-voltage TEM using whole-mount unembedded cultured cells. For details on this issue, please refer to Hipkaeo & Kondo (2016), Hipkaeo (2023), and Chomphoo *et al.* (2024).

Development of procedures for specimen preparation for TEM.

The first successful application of TEM to cell ultrastructural analysis was by observation of whole-mount cultured cells without embedding (Porter *et al*. 1945), That attempt seemed to have done 'unavoidably' rather than 'with distinct reasons' as follows: The limited penetration power of electrons in TEM and the ease with which they are scattered by atoms in cells, required biological (bio-) specimens of unusual thinness, which was estimated at 0.08 μm or less, far less than the > 1 μm thickness possible utilizing the microtomes then available in LM. Therefore, until appropriate ultramicrotomes were devised and made available, it was usual to focus viewing attention on the thin, peripheral extended portions of cultured cells. Despite this limitation, groundbreaking discoveries were made at that early stage in the development of TEM as described in the next section. The recognition of the plasma membranes of cells and intracellular membranes as morphological entities, however, was possible only after thin sections could be reliably prepared using the newly devised ultra-microtomes.

The development of specimen preparation procedures, such as selections of fixatives, embedding media, and staining of sections, was critical for the eventual success of TEM observation. Regarding the fixatives, the remarkable properties of osmium tetroxide (OsO_4) as a fixative had long been known in LM, and it had been employed as a trial fixative for primitive TEM in the 1930s (Marton, 1934). Porter *et al*. (1945) used OsO_4 as the fixative in the form of vapor in the ultrastructural study of whole-mount cultured cells. The use of buffered OsO_4 with adjustment of pH and osmolarity was subsequently recommended for good preservation of cell and tissue ultrastructure by Palade (1952a). While OsO_4 was long known to be appropriate for the fixation of lipids, glutaraldehyde was later found to be an effective cross-linking agent for proteins, more effective than paraformaldehyde/formalin that was/is often used in LM (Sabatini *et al*. 1963). Glutaraldehyde has since then been extensively employed for the pre-fixation of cells and tissues followed by OsO_4 fixation. This combination provided very stable specimens allowing observation of the cytoskeletal components, especially microtubules, later in the 1960s.

Since the need for a high vacuum (10^{-4} torr) in TEM required the removal of water from bio-specimens, an effective and non-damaging dehydration process was needed. The treatment of cells with phosphorus pentoxide in a closed chamber for a short time (one hour) was known to be more effective than air-drying (this chemical was often used for fixation and dehydration together) (Orr *et al*. 1961). This approach was employed in the study by Porter *et al*. (1945). A much-improved dehydration approach that avoided the water surface tension damage only emerged with the development of commercially available critical-point drying equipment later in the early 1970s. With this new drying method, Porter *et al*., re-examined whole-mount cells using high-voltage TEM and revealed ultrastructural details of the cytoplasmic matrix as briefly explained at the end of the previous section.

The use of any supporting (embedding) media was initially not favored for bio-specimens because it was thought to reduce contrast in the image as a result of the small difference in average electron scattering between the bio-specimens and any possible embedding media. The observation of whole-mount cells was thus performed without embedding, and sufficient contrast was obtained without TEM-typical staining. There were, however, two reasons why embedding was critical to obtain reliable identification of the membranous delineation of substructures initially detected in the whole-mount cells. One was to protect specimens from rupture or structural distortion under high vacuum and electron beams in TEM, and the other was to protect specimens from mechanical stress during ultramicrotome sectioning. These benefits of embedding neglected the original concern of reduced image contrast, a drawback that was/is generally overcome by the use of suitable staining chemicals. However, some intracellular components might fail to give images of sufficient contrast even after staining because they do not have sufficient affinity to the stains used. This difficulty later influenced the recognition of fine strand lattices or networks (originally termed microtrabeculae and their lattices) as distinct ultrastructural features of the cytoplasmic matrix, in the re-examination of whole-mount cells by Wolosewick and Porter (1979). The microtrabeculae, after serious debates on their reality, were later suggested to represent the possible sol and gel status as well as the protein concentration of the cytoplasmic matrix by Kondo (2010).

As the first reliable embedding media with sufficient hardness for ultramicrotome and less polymerization damage, major attention was paid to epoxy plastic materials and methacrylate was first introduced by Newman *et al*. (1949). This resin was employed in most studies reported during the early 1950s. Thereafter, another epoxy resin with the trade name Araldite was introduced by Glauert & Brieger (1956). Araldite sections were found to suffer less degradation during irradiation by the electron beam than those cut from methacrylate. Consequently, Araldite did not produce less contamination within the microscope than methacrylate. A third epoxy resin termed Epon 812 was subsequently introduced by Luft (1961). Epon 812 penetrated specimens more efficiently than Araldite, making it easier to cut ultrathin sections, and has remained to the present day the most popular embedding medium.

An early ultramicrotome employing the thermal advance of specimens against the knife was developed by Sjöstrand at the Karolinska Institute. He used it for analysis of skeletal muscle tissue (1943a, b). The development of ultramicrotomes and knives then progressed rapidly in works done by several research groups in various countries. Use of the mechanical specimen advance in the machines by Porter and Blum (1953) and of the thermal specimen advance by Sjöstrand (1953a) were ingenious solutions that are still used. A method for making glass knives for the ultramicrotome was reported by Latta & Hartmann (1950) and the production of diamond knives was reported by Fernandez-Morán (1956). The knife was fixed at a set position of the ultramicrotome, and the specimen-block holder was mounted on a revolving wheel which brought the specimen in contact with the knife only during the downstroke of the specimen holder. The knife was bypassed during the upstroke of the specimen holder. This "single pass" was a novel device by which damage to the surface of the tissue block, and therefore to the next section, was avoided. A liquid-filled trough was mounted against the knife's edge, which was another important innovation of the ultramicrotome. As the tissue blocks were being cut by the knife, resulting sections began to float on the liquid surface in the trough, which prevented sections from folding and made them spread smoothly.

Regarding the staining of ultrathin sections to enhance the contrast of sectioned cellular targets, the use of solutions containing heavy metals such as Pb and Ur was reported by Watson (1958) and Frasca & Parks (1965).

Disclosure processes of individual substructures of cells by TEM.

In this section, the development of our understanding of individual organelles in the early days of TEM is described. A concise explanation by Satir (2005) is recommended for historical overviewing.

1a. Endoplasmic reticulum (ER).

As Palade (1971) recalled in his essay about the research of Albert Claude, in the early days of TEM, Claude had encountered difficulties on account of the excessive thickness of the specimens, and turned to whole cells *in vitro* flat enough and thin enough to let most of the electron beam pass through. In that pioneering study (Porter *et al*. 1945), a “lace-like reticulum” was noticed throughout the cytoplasm and appeared to be composed of chains of vesicular and tubular forms with relatively low electron density. That paper can be said to mark the beginning of the TEM era in cell biology. The micrographs presented in their article were of relatively high quality, high contrast, and sharp definition, probably due to a combined effect of fixation and staining of cellular membranes by OsO4, and of ‘partial extraction’ of the proteins of the cytoplasmic matrix, which occurred rather accidentally by prolonged fixation and subsequent washing of bio-specimens.

In that epoch-making article, the authors initially stated that the lace-like reticulum was scarce in the 'exo' (not 'endo') -plasmic periphery of the cytoplasm, although that description was later found not accurate. Such intracellular disposition and morphology were well represented by the term 'endoplasmic' reticulum (ER), which was coined by Porter soon after its first recognition (Porter & Thompson, 1948; Porter & Kallman, 1952). Similar analyses of whole-mount cultured cells in TEM were subsequently reported by others (Martin & Tomlin, 1950; Selby & Berger, 1952). Because of the whole-mount observation and resulting *en-face*, but not sectioned, views of most intracellular structures, including ER and the cell membrane, their exact delineation features were not clearly visible, that is, whether their margins were represented by distinct electron-dense lines/membrane entities or not. To resolve this, it was critical to observe cells in ultrathin sections made using the then newly devised ultramicrotomes. A careful comparison of the lace-like reticulum encountered in whole-mount cells with structures at corresponding intracellular sites in sectioned cells of various kinds of tissues confirmed ER as a real entity in cells *in situ* by Palade and Porter (1954) and Palade (1956). Since the rather 3D "reticular" disposition of ER in whole-mount cells was, needless to say, lost in randomly sectioned 2D views of cells, it was not easy to judge which structures in sectioned cells actually corresponded to the "lace-like reticulum" in whole-mount cells. In this regard, it should be noted that the TEM analyses of differentially fractionated cell components greatly contributed to the confirmation of ER as a distinct entity in cells *in situ*. With this methodological combination, Palade and Siekevitz (1956a, b) reached the conclusion that Claude's biochemical fraction already called microsomes (Claude, 1943) corresponded to the *in vitro* version of ER.

Thus, the nature of ER was largely established by the early 1950s, including the presence of smooth- and rough-surfaced ERs. The smooth-surfaced ER was defined as composed primarily of interconnected vesicles and tubules, and the rough-surfaced ER as cisterns associated with numerous dense granules and disposed parallel to one another. Physical continuity between the two types of ER was also noted. The nature of the dense granules (ribosomes) is described in the next section. During the careful TEM observation needed to establish the existence of ER, information about characteristics of cells accumulated in the long history of LM histology was also highly useful. For example, the highly basophilic "ergastoplasm" characterized by high basophilic features was long known to be enriched in protein-secreting cells such as the pancreas and salivary glands as well as in neuronal somata where the structures were called Nissl bodies. The TEM analysis with this LM viewpoint revealed that cellular domains of the ergasotplasm were composed of stacks of rough-surfaced ERs. The finding led to the idea that the rough-surfaced ER are involved in protein production (Porter, 1953). By the same strategy with careful correlation to accumulated information using LM, the smooth surfaced ER was considered to be involved in lipid metabolism based on its remarkable enrichment in various lipoid-producing cells such as hepatocytes (Palade & Siekevitz, 1956a), adrenal cortical cells (Ross *et al*. 1958), sebaceous gland cells (Palay, 1958), the ovarian and testicular interstitial cells (Muta, 1958; Christensen & Fawcett, 1960), and retinal pigment cells (Yamada *et al*. 1958; Porter & Yamada, 1960).

ER was found more or less evenly distributed throughout the cytoplasm, but not confined to the endoplasm, of sectioned cells from the nucleus in the cell center to the peripheral cell membrane in most cell types. Palade (1956), commented that the name "endo"-plasmic reticulum would be a temporary one until a more appropriate name, descriptive of the morphology or preferably the physiology of the ER could be found. This name "endoplasmic" is, however, still employed at present.

At Sweeden's Karolinska Institute, Sjöstrand published studies on the ultrastructure of the mouse pancreas, with thin sections cut using a locally developed ultramicrotome (Sjöstrand 1953a). He demonstrated that the cytoplasm was filled with concentrically arranged and densely packed bio-membranes, to one side of which electron-dense particles were attached. His electron micrographs were regarded as of the world's highest resolution at that time (Sjöstrand, 1953c, 1954). Although no specific names were given to the membranes in those studies, they evidently corresponded to the rough-surfaced ER that Palade reported almost simultaneously as cited above. In addition to those studies, Sjöstrand performed pioneering TEM studies on skeletal muscles (Sjöstrand, 1943a, b) and retinal rod cells *in situ* (Sjöstrand, 1948) in sections made using his own prototype ultramicrotome. The TEM studies by Sjöstrand was thus done from the beginning as the observation of cells *in situ* in sections, without passing through observation of whole-mounted cultured cells, different from Porter and the Rockefeller group. Instead, his studies were based on a lot of his former observations of the cytoplasm using polarization microscopy. In addition, the exceptionally high resolution of his micrographs helped the general acceptance of his findings.

As a result, an idea began to appear even in the early days of TEM based on those findings that the ER

was a continuous network of membrane-bound cavities extending throughout the entire cytoplasm and surrounding the nucleus which it surrounded with a discontinuous 3D moat (Palade, 1956). This implied that the ER should not be considered a simple organelle, but rather a complex system in which various organelles and apparatus are integrated. That idea is quite in concert with the recent inter-organelle communication (Dolgin, 2019). In fact, an intimate apposition of ER to mitochondria is found in Palade's article (1956, refer), though no special notes were made about it by him.

1b. Sarcoplasmic reticulum (SR)

This structure was reported as a specialized form of ER in muscle cells in the early days of TEM by Bennett & Porter (1953). In that study, the authors considered the SR to be "rediscovered" because it was surprisingly recognized and reported in LM already in the 1800s. Dobie (1849) first noticed a component of the sarcoplasm as a homogenous membrane stretching between myofibrils, which had a relationship to the cross-striations of skeletal muscles of fish and amphibia. Intracellular transverse and longitudinal components equivalent to SR were first visualized definitively in skeletal muscles impregnated with gold by Thin (1874). His report was followed by Melland (1885), Cajal (1888), Marshall (1888) and Retzius (1890). Thereafter, it was Veratti (1902), a pupil of Golgi, who provided the most extensive and valuable description of SR including the special relationship of portions of the reticulum to the sarcolemma and Z band in skeletal muscles of various vertebrates and invertebrates treated with metallic impregnation. The TEM "rediscovery" of the classical LM findings was concisely summarized by Bennett (1956b) which included the classical (1800s) literature references cited above.

Those intracellular structures identified in the classical LM observations, equivalent to the now termed SR in TEM, were long ago suggested to transmit an excitatory impulse from the sarcolemma to myofibrils deep within the muscle fiber. That suggestion is amazing, coming as it did half a century before Huxley & Taylor (1955) brought forth evidence, compatible with the suggestion, indicating that there was a local transverse conducting system within the muscle fiber.

2. Ribosomes

As described in the previous section on the ER, its rough- and smooth-surfaced profiles were already distinguished when the ER was confirmed to exist in cells *in situ*. The rough-surfaced profiles were ascribed to the association of 'small electron-dense granules' or 'particles of Palade' (Palade, 1953b, 1955) or 'opaque particles' (Sjöstrand, 1954) with the ER membranes. They were recognized as one form of the small granules/particles, which were later named ribosomes. The other form of ribosomes was almost simultaneously recognized as being dispersed in the cytoplasm without association with the ER membranes.

Because of their granular nature and lack of a limiting membrane as well as their tiny size rendering them below the LM resolution limit, it was debated whether ribosomes were real or artifactual. It was already known that fixatives, especially OsO4, and staining of sections with heavy metals could result in random deposits of tiny structures that were not present in the original bio-specimens. How was the reality of ribosomes eventually determined?

Criteria for recognizing that structures are real and not artifactual have been/are pointed out empirically. Two of these criteria are 1) the consistent and reproducible occurrence of a given structure regardless of preparation procedures employed and cell type examined and 2) its consistent appearance in ways compatible with any known patterns/laws of materials in physics. We can also add that regularity of pattern is often a feature of genuine structures, requiring high-quality TEM images to demonstrate this. If allowed to say ultimately, 'Beauty is Truth, Truth Beauty (by John Keats)' may be the sole criterion for the reality in TEM. This is why TEM photos with high quality are always required for reliable data for us.

Indirect evidence was gradually accumulated in support of the reality of ribosomes (Palade, 1953b, 1955; Sjöstrand, 1954). One line of evidence was obtained by observation of bio-specimens after aldehyde fixation without the utilization of OsO_4. Aldehyde fixation was empirically known to induce artifactual precipitates to a far lesser degree than OsO_4. Although the cells appeared poorly preserved in aldehyde-fixed specimens, such granules/particles of appropriate sizes were constantly found in expected numbers and locations in the cells. In addition, careful observation of specimens fixed with OsO_4 revealed consistency in the intracellular distribution and population densities of the granules/particles in various cell types including adult differentiated cells, embryonic or proliferating cells, and exocrine cells. The small granules/particles were found in all cell types except for the adult erythrocyte, and their number varied considerably from one cell type to another but seemed relatively constant for any given type. The highest density of membrane-free granules/particles was seen in embryonic cells and rapidly proliferating cells of adults, while that of rough-surfaced

ER was seen in the basal domains of exocrine gland cells and in neuronal LM domains termed Nissl bodies. All of these cells and cell domains were known to be basophilic and to be often termed ergastoplasm in LM (Deane & Porter, 1960). In the outside surface of rough-surfaced ER membranes, when tangentially sectioned, the small granules/particles were frequently localized in linear series and spaced at more or less regular intervals on the outer surfaces of rough ER membranes. Sometimes the particles formed consistent patterns, among which parallel double rows, loops, spirals, circles, and rosettes were predominant, all patterns of distribution now known to be typical of ribosomes. Those findings were finally able to rule out the possibility that the granules/particles were artifacts occurring during specimen fixation and preparation.

A small particulate component of comparable size was identified in homogenates of fresh, unfixed cells of various types using differential centrifugation. Biochemists showed that these particles contain a high level of ribonucleic acids by biochemists (Barnum & Huseby, 1948; Petermann *et al*. 1952), which was a point to later clarify the function of the small electron-dense granules/particles, subsequently. The particulate structures were thus termed ribosomes subsequently. The ribosomes were finally confirmed as macro-molecular machines within all cells that perform protein synthesis.

A well-organized combination of careful observation with orthodox morphological disciplines in LM and TEM, as well as biochemical analysis with the differential centrifugation from tissue homogenization contributed much to the recognition of new organelles in TEM. In this regard, the TEM research group at Rockefeller Institute was important, because it included an active group of biochemists headed by Claude who was a pioneer in the application of TEM to the life science of cells. Palade (1971) regarded Claude as the father of the cell biology.

3. Golgi apparatus and secretory vesicles

This organelle, sometimes termed the "post office of the cell", was first described more than 100 years ago by the Italian doctor and pathologist Camillo Golgi (1844-1926), who was known for his studies on the nervous system and received the Nobel Prize in physiology and medicine in 1906, shared with Santiago Ramon y Cajal, the Spanish histologist. He described this reticulated structure as consisting of anastomosing ribbon-shaped filamentous elements, small plates with a clear center that served as the nodal points of the reticulum, and rounded discs. He hypothesized that this structure might be related to secretory function, and more broadly to cell nutrition because the LM had demonstrated very early that this organelle was well developed in secretory cells (for details, refer to Mazzarello & Bentivoglio, 1998).

Until the mid-1950s, the reality of the Golgi apparatus had been controversial. Some non-believers considered that the metal impregnation methods with silver or OsO4, which Golgi and others used to demonstrate the structure, produced artifactual depositions of heavy metals on different cell structures in different cell types. Even Palade and Claude, who were given the Nobel Prize in 1974 for their studies essentially based on the Golgi apparatus function, originally suggested that the Golgi apparatus represented one or more myelin figures that emerged artificially during the preparation of cytological samples (Palade & Claude, 1949 a, b). In TEM of whole-mounted cultured cells (Porter *et al*. 1945), dense bodies characterized by rather angular outlines appeared consistently. In some cells, the dense bodies were grouped near the nucleus, while in others they were scattered and fewer in number. It was then concluded that the structures were Golgi apparatus by their size, distribution, and seemingly osmiophilic properties. Because of their density after osmium staining, however, any exploration of their internal structure, if any, was not possible. Thereafter, several attempts were made to look for ultrastructural equivalents to the LM silver-impregnated Golgi apparatus in ultrathin section TEM by several researchers. In the early1950s, several research groups independently obtained evidence for the reality of the Golgi apparatus (Dalton, 1951, 1952; Dalton & Felix, 1954, 1956), (Sjöstrand & Hanzon, 1954a, b); Burgos & Fawcett, 1955; Yamada, 1955a; Weiss, 1955). In short, TEM images of sufficient resolution revealed that the Golgi apparatus consisted of membrane-delineated lamellae, vesicles, and vacuoles. It was further noted that the deposition of metallic osmium occurred in its lamellar components, indicating that these exactly correspond to the classical Golgi body recognized in LM using metallic impregnation methods. As a result, there were no longer any doubts about the existence of the Golgi apparatus.

4. Mitochondria

Unlike ER and ribosomes which had not been discerned using LM, mitochondria had already been known to exist in living cells since the mid-19th century. In LM, mitochondria were recognized as granules or often elongated bodies of filamentous or rod-like shape with active and passive movement and the potential for division. Differential centrifugation was able to isolate mitochondria, although no interior structure was discerned (Claude & Fullam, 1945; Claude, 1947).

In the first TEM study of whole-mount cultured cells cited in previous sections (Porter *et al*, 1945), mitochondria were identified as mostly electron-dense thread-like entities of various lengths and fairly constant width, or as occasionally spherical or vesicular forms. Distinct evidence for the mitochondrial limiting membranes (inner and outer) and the intra-mitochondrial granules was obtained by observation in ultrathin sections independently by Palade (1953a) and by Sjöstrand (1953b) almost at the same time.

Palade and Sjostrand initially differed in their interpretations of the membrane organization. Palade initially reported a single mitochondrial (corresponding to the present inner mitochondrial membrane), and that the intra-mitochondrial membranes (corresponding to the present cristae membranes) were folds of the former membrane (Palade, 1952b). This implies the intra-fold space (corresponding to the present intra-crista space) is continuous to the extra-mitochondrial cytoplasmic space. In his subsequent article, Palade (1953a), however, noted that the mitochondria-delineating membrane "appears to" be "double" with TEM images showing a double membrane in places. This later interpretation is close to the present understanding of mitochondrial structure. The article by Sjöstrand (1953b), which was submitted in 1952 and published a few months earlier than Palade's article of the same year (Maunsbach, 2008a), stated that mitochondria were bordered by an 'outer' double-edged membrane which appeared as two electron-dense lines (corresponding to the present outer and inner mitochondrial membranes) separated with a fairly constant distance (corresponding to the present intermembrane space). In short, the present outer and inner membranes were together treated as a single distinct membrane. Sjöstrand also reported that, in the interior of mitochondria, a double-edged 'inner' membrane appearing as two electron-dense lines (the present crista membranes and intra-crista space) were oriented perpendicularly to the long axis of the organelle. One end of the inner membrane (the portion of the present crista continuous with the present inner mitochondrial membrane) was in contact with the 'outer' surface membrane (the present inner mitochondrial membrane), while the other end was free. The two lines (the present cristae membranes) met at both ends of the membrane thus delimiting a narrow space (the present intra-crista space) between the two lines. The interpretation of ultrastructural features by Sjöstrand was from the beginning almost the same as the presently defined mitochondrial features except for the negated continuation of the intra-crista spaces with the intermembrane spaces (for a comprehensive review, refer to Sjöstrand, 1977). Considering the technical limitation of TEM and specimen preparation, the analyses by these two scientists are retrospectively of a very high standard. In addition, both Palade and Sjöstrand reported the occurrence of intramitochondrial dense granules or opaque spherical bodies measuring 18-40 nm in diameter, which correspond to mitochondrial matrix granules in the present terminology.

5. Synaptic vesicles

The 'reticular theory' was proposed first by Joseph von Gerlach (1820-1896) in Germany to describe the properties of neurons. This theory stated that the nervous system was composed of a continuous network of neurons connected to and dependent upon one another. With the LM methods then available, neuronal cell bodies surrounded by a network of neuronal extensions (axons and dendrites) without a definite beginning and end were visible. Following his proposal, Camillo Golgi in Italy created 'the black reaction', a silver-impregnation staining method for the nervous tissue (Golgi, 1873), and demonstrated the complete form of neurons as well as their cellular extensions. Golgi strongly supported and enunciated the reticular theory. On the other hand, Santiago Ramón y Cajal in Spain, also employing Golgi's black reaction, proposed the neuron doctrine, in opposition to the reticular theory. The latter theory stated that individual neurons developed and existed as independent and discrete units. The two different theories were much debated at the turn of the twentieth century. Both Golgi and Cajal shared the 1906 Nobel Prize in Physiology or Medicine. For historical details on this matter, please refer to Cimino (1999) and de Carlos & Borrell (2007).

It finally became evident by TEM analyses that in the nervous system of both invertebrates (Robertson, 1953) and vertebrates (Palade & Palay, 1954; de Robertis & Bennett, 1954, 1955; Palay, 1956), two neuronal elements (presynaptic and postsynaptic elements) were separated from each other by a gap at the synapse (synaptic cleft) of only about 20 nm. In other words, there was a protoplasmic discontinuity between the pre- and postsynaptic elements, each of which was surrounded by its own cell membrane. This TEM feature of the synapse was a strong confirmation of the neuron doctrine enunciated and defended by Cajal against the reticular theory proposed by Golgi.

It was further recognized using TEM that within the presynaptic element (the axon terminal or bouton), an accumulation of vesicles termed synaptic vesicles was present. These were located close to the presynaptic membrane.

Shortly before the relevant TEM reports, transmitter release at the frog neuromuscular junction was found to induce postsynaptic miniature end-plate potentials that were ascribed to the release of discrete packages of neurotransmitter (quanta) from the presynaptic nerve terminal (Fatt & Katz, 1950a,b). It was further hypothesized that the vesicles, by a secretory mechanism, would release their contents into the synaptic cleft (vesicle quantal hypothesis) (del Castillo & Katz, 1954a,b). Palay (1956) went further to propose that the vesicles visible by TEM were the structural source of the miniature, spontaneous pulses reported by del Castillo & Katz (1954a,b). Thus, the hypothesis of quantal transmitter release eventually had a structural correlation. The identification of synaptic vesicles was thus a nice example of a previous hypothetical consideration in physiology being confirmed by morphological evidence in TEM.

6. Lysosome

Lysosomes were first identified, along their enzymatic properties, in liver homogenates by Christian de Duve (1955), and were subsequently shown morphologically in TEM by Novikoff (1956). This means that, as a rather exceptional case, the identification of this organelle was established by the preceding biochemical speculation of its existence.

To purify and isolate glucose 6-phosphatase, an enzyme critical for regulating blood sugar levels, de Duve employed cell fractionation and detected the enzyme activity in the microsomal fraction. He then found the enzyme activity to increase remarkably after the fraction had been refrigerated for five days. To account for this, he and his coworkers predicted the existence in this fraction of sac-like structures containing the enzyme and enclosed by semipermeable membranes. For the supposed particles, he proposed the name lysosome (de Duve *et al*. 1955; de Duve, 2005). Subsequently, in a TEM study of the cell fractions, Novikoff *et al*. (1956) revealed the presence of hitherto undescribed 'dense bodies' that fit these predictions. These bodies were 0.37 μm in mean size and contained tiny electron-dense granules in the internal cavities enclosed by limiting membranes. Using a staining method for acid phosphatase in LM and TEM, they confirmed the location of the hydrolytic enzymes of lysosomes. de Duve won the Nobel Prize in Physiology or Medicine in 1974 for this discovery, together with Claude and Palade.

Bennett (1956c) suggested that lysosome granules may contain materials taken into liver cells by phagocytosis or pinocytosis, based on his experience that particles of carbon and colloidal dye, when injected intravenously in frogs, were found to be deposited in granules resembling the lysosome bodies reported by Novikoff *et al*. (1956).

In the following years, a wide variety of vesicles, some of which contained engulfed cytoplasmic material, were discovered using TEM. These particular vesicles were identified as pre-lysosomes (Smith & Farquhar, 1966). Pre-lysosomes were further found to form *de novo* in the cytoplasm from a cup-shaped membrane termed a phagophore, which eventually led to the concept of autophagy, for which Yoshinori Ohsumi became a Nobel laureate in Medicine and Physiology in 2016 (Ohsumi, 2016).

7. Nucleus

Needless to say, the cell nucleus was long known to exist based on LM observations. In the early days of TEM, it was difficult to discern any internal structure of the nucleus, in contrast to the cytoplasm, because of the presence of an enormous quantity of chromatin and ribonucleoproteins and the absence of intra-nuclear membranes isolating the individual components.

There had long been an idea that a nuclear membrane must exist through which the interphase nucleus could exert fundamental control over such complex cytoplasmic activities as protein synthesis. It was TEM which presented clear evidence that the nucleus in interphase does possess an enveloping, membranous sheath. The early TEM studies were made on nuclear membranes teased from the cell without sectioning and disclosed pores in the nuclear envelope (Callan & Tomlin, 1950; Bairati & Lehmann, 1952; Harris & James 1952; Gall, 1954). Subsequent thin-section analyses by several researchers clearly showed the nucleus to be bounded by two (outer and inner) membranes with pores at their fusion sites, which was close to our present knowledge of the nuclear envelope (Hartmann, 1953; Bahr & Beermann, 1954; Rhodin, 1954; Watson, 1954, 1955; Afzelius, 1955; Palay & Palade, 1955). Watson (1955) mentioned that in certain cells, the outer nuclear membrane was continuous with membranes of the ER. Hence, the perinuclear space was continuous with cavities enclosed by those membranes, at least in a transitory way. Based on those observations, he proposed two hypothetical pathways of exchange between the nucleus and the cytoplasm: by way of the perinuclear space and cavities of ER and by way of the pores in the nuclear envelope.

8. Plasma membranes/Cell membranes

Until the end of the 19th century, the existence of

cell membranes had been questioned, although some inferred that cell membranes must exist in animal cells to confine intracellular component movement. Cell membranes gained more attention when studies on osmosis and permeability were active. Overton (1895; refer to Kleinzeller, 1999) proposed that cell membrane was made of lipids, and Gorter & Grendel (1925) proposed the lipid bilayer hypothesis. Attempts were made to extract the lipid from human red blood cells and to measure the surface area that the lipid would cover when spread over the surface of water. As a result, the cell membrane was confirmed to contain a lipid bilayer. Thereafter, Danielli & Davson (1935) proposed a model for the membrane structure termed the "pauci-molecular model". According to this, all biological membranes consist of a sandwich of protein-lipid-protein in which a lipoid center is surrounded by mono-layers of lipids that are covered by protein mono-layers. This model dominated cell membrane studies for the following 30 years, until Singer and Nicolson (1972) proposed the "fluid mosaic model" which is the dominant model now. In the new model, while still retaining the lipid bi-layer concept, proteins are regarded as globular entities embedded in a mosaic in the membrane instead of as mono-layers on the surface. For more detailed information, please refer to Gerald (2009). In summary, the cell membranes were seen but mostly disregarded as an important structure with cellular function until the mid-20th century when TEM was introduced into the biosciences.

The clear recognition in TEM of bio-membranes (including the plasma membranes and intracellular membranes) as entities distinct from the surrounding cytomatrix was not fully successful in the observation of whole-mount cultured cells in the early TEM work by Porter *et al*. (1945). For bio-membranes to be better understood, ultramicrotomy-made sections of cells had to be examined. In specimens fixed with OsO4, which was rather common in the early day TEM, the bio-membrane usually appeared as a single electron-dense line, although it occasionally exhibited an intermittent doublet of dense lines with an intermediate thin electron-lucent zone. The doublet of dense lines was more consistently seen after the employment of potassium permanganate as an alternative fixative (Robertson, 1957), or the *en bloc* staining of specimens with uranyl acetate. Robertson noted that the thickness of each of the two dense lines was about 2 nm and that of the electron-lucent dense zone about 3.5 nm, hence approximately 7.5 nm overall. These features were compatible with the membrane models derived from biochemical analyses such as the model of Danielli & Davson (1935) and that of Singer and Nicolson (1972).

Although the composition of a bilayer of amphipathic lipids for the bio-membrane was generally accepted from the beginning of TEM, differences in the thickness between the plasma membranes and the intracellular membranes were often noted. Sjöstrand (1954) was a pioneer who paid attention to the membrane thickness and performed detailed measurements on his micrographs which were exceptionally high quality for the time. According to the measurement data in his study, the thickness of plasma membranes mostly appearing as a single dense line was 6 nm, that of ER membranes was 4 nm, and the mitochondrial membranes were 3.5 nm, implying that the plasma membranes were significantly thicker than any intracellular membranes. Slightly later, Yamamoto (1963) also measured the thickness of various bio-membranes utilizing a densitometer on TEM micrographs. He classified bio-membranes into two groups in terms of thickness: the thicker group included synaptic vesicles, multivesicular bodies, and Golgi vesicles as well as the plasma membranes, and the thinner group included mitochondrial and nuclear membranes, Golgi lamellae, and endoplasmic reticulum. Membranes in the latter membranes were 85-90 % of the thickness of those in the former. Such findings led cell biologists in the 1960s to consider that the membranes of the former group have an intimate functional relation or contiguity with the plasma membranes, discrete from those of the latter.

In this regard, of note is a formerly published study by Bennett (1956a). In that study, Bennett first introduced the notion of Palade (1953b, 1955): by showing extensive infoldings of the cell membrane and their frequent appearance close to isolated vesicles in the cytoplasm, and by referring to the classical paper on pinocytosis by Lewis (1931), Palade suggested that the vesicles might have been formed from the pinching off of a recessed tip of the fold or that the vesicles might be on the process to coalesce with the fold. In concert with the notion of Palade, Bennett bravely introduced the hypothesis in the study that "membrane flow" may be an important part of a type of active transport mechanism carrying particles within, into, and out of cells. The hypothesis by Bennett is largely compatible with the presently well-known "membrane trafficking" idea (More *et al*, 2020). Palade and Bennett must have been induced by the finding that the ER is a continuous network of membrane-bound cavities permeating the entire cytoplasm in whole-mount cultured cells by Porter *et al*, (1945). When considering the hypothesis and idea by Bennett and Palade together with the inference from the membrane thickness finding of Yamamoto explained in the above paragraph, it cannot be negated that the original membrane flow was developed/matured into a more refined entity with two distinct directions, eventually leading to the present version of "membrane trafficking".

Cytoskeleton

The possible existence of a cytoplasmic apparatus controlling the relative positioning of various morphoplasm elements seems to have been considered during the first half of the 19th century. The presence of "glutinous matter" inside amoeboid organisms was described by Dujardin (1835; refer to Szent-Gyorgyi, 2004), while a viscous protein was extracted from muscles using a concentrated salt solution by Kühne (1864; refer to Szent-Gyorgyi, 2004). The protein was termed "myosin" and considered as responsible for the rigor state of the muscle. Lohmann (1934) found that ATP was likely to be the energy source for the contraction of muscles, and Engelhardt & Lyubimova (1939) reported that myosin had ATPase activity. Actin, discovered by Straub (1943), was found to constitute the contractile system together with myosin and ATP. It was Bowman (1840) who examined skeletal and cardiac muscles of a variety of animal species using LM and formulated the foundation of the current understanding of the striated muscle structure.

Much progress was made in understanding the mechanism of muscle contraction in the early 1950s. Hanson & Huxley (1953) and Huxley & Hanson (1954) provided the first direct proof in LM that the A bands contained myosin: when they extracted myosin from myofibrils, the A bands disappeared and the myofibril was no longer striated, leaving only a "ghost" with a faint "backbone" with Z disks still intact. A surprising feature of these "ghost" myofibrils was that the sarcomere lengths were unchanged. These authors confirmed the LM finding in TEM. It was concluded that the A bands contained thick filaments, ~15 nm in diameter, and that these filaments disappeared when myosin was extracted. The A and I bands contained thinner filaments, ~7 nm, and the filaments were thought to be composed of actin. In addition, extraction of both myosin and actin from myofibrils resulted in a "skeleton" myofibril still containing Z disks in sarcomeres of unchanged length. This novel information led Huxley and Hanson to propose the sliding filament model of contraction. The sliding filament theory was independently introduced also by Huxley & Niedergerke (1954) using interference microscopy. There were numerous comprehensive studies on the general ultrastructure of striated muscles in the 1950s by researchers using TEM represented by Pease and Baker (1949), Bennett & Porter (1953), and Hodge *et al* (1954).

Other than myofibrils, it was long difficult to identify with certainty actin filaments within cells using TEM as entities discrete from tonofilaments whose existence was known in some epithelial cells in LM. This difficulty was because of the low electron densities of components composed only of proteins. Biochemical studies had shown that heavy meromyosin bound specifically with actin, making it possible to detect free actin-filaments in the cytoplasm of almost all cells independent of the striated myofibrils, although this was done much later than the early days of TEM (Ishikawa *et al*, 1969). Since then, actin filaments have been classified as microfilaments having a diameter of 7 nm, whereas the epithelial tonofilaments were identified as intermediate filaments discrete from microfilaments based on their thickness of 10 nm (Ishikawa *et al*, 1968).

Microtubules were first identified within cilia in TEM (Manton & Clarke, 1952; Fawcett & Porter, 1954) although the resolution of their micrographs was initially somewhat limited by poor specimen preservation. Prefixing with glutaraldehyde followed by osmium tetroxide greatly improved cell preservation, as explained in the previous section. Cytoplasmic microtubules were depolymerized by cold, the standard temperature for osmium tetroxide fixation, and were not seen distinctly until glutaraldehyde fixation was introduced. Since then, the ubiquitous presence of microtubules has been revealed in almost all eukaryotic cells, and the architecture of the 13 globular subunits in microtubules was recognized by Ledbetter & Porter (1964).

Development of Journals and Societies related to Electron Microscopy

New findings and concepts in any field of science would be just a cloud of dust unless they are published in appropriate Journals. In the early days of TEM development in life sciences, two kinds of problems were apparent in getting findings published. First, the quality of reproduction of TEM images, needing to be of higher quality than for LM images, was often criticized as disappointing in journals such as the American Journal of Anatomy and the Journal of Experimental Medicine in the early days of TEM.

Another problem related to the editorial policies of existing journals, whose boards of editors could not always recognize the significance of discoveries or new concepts appearing in manuscripts parading before their eyes. A story is told about the study of the ultrastructure of cilia by Fawcett & Porter (1954). They reported for the first time the "9+2" arrangement of pairs of tubule-like structures seen in cross sections of cilia and also of the filamentous nature of ciliary rootlets and the associated cytoplasmic matrix. These findings have been retrospectively regarded as a major milestone that foreshadowed recognition of the

"cytoskeleton", which now figures prominently in the cell biology literature. Despite being such an important study, the manuscript was rejected by the editors of the Journal of Experimental Medicine in the fall of 1953, largely on the basis that the study was too descriptive and lacked a major experimental component. It was also clear that the editors did not wish to publish papers describing normal morphology. However, the study reported a system for motility not based on actin and myosin, and it eventually came to provide essential background for an understanding of Kartegener's syndrome caused by abnormal cilia. In other words, it can be stressed that normal things have to be discovered and described before their pathology can be recognized. Although this happened 70 years ago, it is still the case that morphological study is given a low priority in the present day when molecular biology is powerful. Present morphologists encountering difficulties in funding and publication should keep this in mind. Eventually, the prominent pioneers of TEM got together to urge the establishment of a new journal. This led to the successful founding of the Journal of Biophysical, Biochemical, and Cytology (JBBC) in 1955, published by The Rockefeller University Press. The name was later changed into the Journal of Cell Biology (J Cell Biol) in 1962. Because of the prosperous history of JBBC/JBC after the start of its establishment until now, their positive and brave decision to the new Journal founding against the general trend in the early days is retrospectively regarded as correct in terms of science development. The Electron Microscopic Society of America was founded in 1942 and it is now termed the Microscopy Society of America. For more details, please refer to Porter and Bennett (1981).

In Sweden, Manne Siegbahn, a Nobel Laureate in Physics and head of the Nobel Institute for Physics in Stockholm, planned to build a TEM in the later 1930s as inspired by the developments by Ernst Ruska in Berlin. Sjöstrand was engaged in this project already in 1938 as an assistant with the aim of applying the new instrument in life sciences. After a while, Sjöstrand used it to record for the first time skeletal muscle fibers in thin sections produced by cutting away wedges from thicker sections. He reported his observations in a short paper, which Manne Siegbahn sent to Sir Henry Bragg in London, who in turn submitted the paper to Nature (Sjöstrand, 1943a, b). In 1947, he spent some time at the Massachusetts Institute of Technology, Francis O Schmitt's laboratory in the USA. After returning from the US, he installed a TEM in his Anatomy Department at the Karolinska Institute in 1949 and was highly active in the publication of many new findings of high-resolution electron micrographs by means of his own constructed ultramicrotome with the thermal advance, in contrast to the mechanical advance by the Rockefeller group. Most of his micrographs are objectively superior to that of the Rockefeller group as exemplified in a study of mitochondria (Sjöstrand, 1953b). This study was first presented at the annual meeting of the Electron Microscope Society of America in 1952 and led to intense discussion with his US competitors. Using his improved technique, Sjöstrand also obtained the first high resolution TEM micrographs of neural myelin sheaths and clarified the lamellated organization of chloroplasts (for more details and references, refer to Maunsbach, 2008b). As a result, he also encountered the necessity for a new journal accepting TEM data like the US situation described above. In addition, it seems to be likely that he wished to compete with the US activity. Consequently, the first issue of the Journal of Ultrastructure Research was published in 1957 by the Academic Press. This journal, which changed its name to the Journal of Structural Biology in 1990, continues to contribute much to the structural biology field. The Scandinavian Society for Electron Microscopy was established in 1948, and the first European Regional Conference on Electron Microscopy was held in Stockholm in 1956 (Lindberg & Nilsson, 2010).

Development of Electron Microscopy in Japan representing Asia

Almost all descriptions above are about the development of TEM in the USA and some European countries. It is natural for us, Asian scientists, to wish to know how much our Asian predecessors contributed to the development of TEM in life sciences. What follows is a summary of TEM development in Japan, an Asian forerunner country in many fields of sciences.

Due to its defeat in World War II in 1945 and the resulting financial and social collapse, the development and application of TEM to life sciences in Japan was delayed. Nevertheless, ultrastructural analyses of some biological specimens were performed in the 1950s using ultramicrotome and TEM, both of which were made in Japan (e.g. Shimazu magnetic model SM-1B and -2, and Hitachi HU-10) (Yasuzumi, 1951, 1956a, 1956b; Yasuzumi *et al.*, 1952, 1953; Kajikawa, 1959; Kajita, 1959).

Soon after ease of restrictions on foreign travel imposed by the alley's military regime that temporarily governed post-war Japan, several scientists went abroad for one or two years, mainly to the US, to learn about the biological TEM. These scientists published actively in the 1950s. They were represented by Yamada E who contributed to the original establishment of the Golgi apparatus as an ultrastructural entity (Yamada, 1955a, 1955b) and who was included as a member of the congenial

and collegial group in the world development of TEM by Porter and Bennett (1981). In the 1960s and 1970s, an increasing number of anatomists and pathologists as well as bacteriologists in universities working with TEM across Japan went abroad for direct scientific communication.

The Japanese Society for Electron Microscopy was organized in 1949, and the Journal of Electron Microscopy was founded as its official Journal in 1952. The Society and Journal changed their names to The Japanese Society for Microscopy, and Microscopy, respectively, in 2002 (Japanese Society for Electron Microscopy, 2002).

Scientific societies in Japan have long had the custom of translating almost all Western scientific terminologies into Japanese. This was the case also for TEM and LM, and it has long been usual for university students to learn any histological term using both Japanese and Western (German before and English after World War II, and even Latin) terminologies. There is one exception to this: endoplasmic reticulum (ER). As explained in the previous section, the word "endo-plasmic" is theoretically wrong, and its Japanese name does not come from its literal translation but instead means "vesicular apparatus". Although it does not include its original meaning of "reticulum" in a strict sense, the Japanese name is regarded as providing a more correct description than its Western counterpart. Almost all Japanese scientific societies believe that their translated terminology, in addition to the original Western terminology, is useful for catching exact meanings and developing science in Japan. There is an opinion that this belief may have contributed to the increase in the number of Nobel laureates from Japan after World War II. Other Asian countries may imitate such a Japanese way.

CONCLUSION

Several lessons emerge from this review. One of them is that discoveries/developments in the early days of TEM from the mid-1940s till the 1950s were made not by a single brilliant researcher, but by a considerable number of competing researchers, mainly in the USA and Sweden, countries that had suffered the least societal damages in World war II. Competition was particularly strong between those within the Rockefeller Institute of the USA and those at the Karolinska Institute, Sweden.

It was by no means accepted in the mid-1950s that what one saw with TEM had any bearing on evolution, biochemistry, or cell physiology as described in the Harvey Lecture by Porter KR in 1956 (Satir, 2005). Researchers at both institutes mentioned above exhibited strong wills and dedication to progress toward the success in the application of this new methodology, TEM, in the life science field. Claude, Palade, and Porter at the Rockefeller Institute and Sjöstrand at the Karolinska Institute were particularly eminent. However, Nobel prizes were given only to Claude and Palade as well as de Duve in 1974. It is a shame that Porter and Sjöstrand, especially the former, did not receive Nobel medals, although the two concentrated on the analysis of ultrastructural morphology, while Nobel laureates Claude and Palade combined ultrastructural morphology with biochemistry. Perhaps the prevailing bias against pure morphology had influenced the Nobel committee, reminding of a similarity to the bad experience of Fawcett and Porter which ignited the new establishment of J Cell Biology as explained in the previous section.

Another lesson from this review is that morphological methodologies (i.e. TEM) were built on the training and interpretation based on existing methods. Palade provided an interesting comment in his article about the career of Albert Claude (Palade, 1971): "*Claude was entering the TEM field with the point of view of a cytologist who had had already a long period of experience with animal cells in tissues and in culture. He knew and respected the cytological literature of the light microscope era and his intent was to use it as a foundation for future developments, rather than to disregard it. He had a clear understanding of what was essential and what was questionable in histological techniques and was willing to experiment extensively using these techniques as a starting base. Finally, he believed in gentle, careful handling of biological specimens, and had an innate feeling for biological structure, which he expected to be, at the newly explorable level (or at any level), a 'wonder of creation."* His comments are represented by what is explained about ER and SR in this review including the importance of classical literature related to the subjects under consideration. This lesson is, in a sense, representing the famous European metaphor, "standing on the shoulders of giants—*nani gigantum umeris insidentes*".

The other lesson is that it was important to pay attention to related/adjacent research fields, such as cell fractionation and cell culture in the early days of TEM, and to employ them when necessary for advancement and success. As Nobel laureate Brenner (2002) wrote, "*the progress in science depends on new techniques, new discoveries by hard work and new ideas based on discoveries, probably in this order*".

From a different viewpoint, here is an interesting point worth noting in terms of the attitude to grow young scientists as a mentor. TEM pioneers such as Sjöstrand

and Bennett authored a rather limited number of published articles. According to Maunsbach (2008b), Sjöstrand had a policy of not putting his name on his students' or visitors' articles just because the work was done in his lab, using his grants and involving discussion with him. As a result, he was usually a sole author. The same seemed to be the case for Bennett. Several Japanese researchers spent one or two years in Bennett's laboratory and later became leaders in the Japan TEM field: their papers were authored only by themselves without Bennett. Although the policy of mentorship may be different among various scientists, this policy of the two pioneers is quite impressive and admirable to the present researchers considering the recent trend in the scientific publication of excessive attention paid to the number and statistical evaluation scores given to articles.

Finally, the literature from Western prominent pioneers in ultrastructural analyses of cells in the early TEM days seems to exhibit essential elements in the spirit of science. Bennett (1985) stated that such an essential element was eloquently expressed by the great Japanese poet named Basho, master of the "Haiku" (Japanese traditional short poem), who brought out his wisdom, his sensitivity, and his perceptions in profuse outpourings of prose and poetry during the late seventeenth century. When Basho was asked by one of his pupils what should one do to become a great 'poet' (please replace with 'scientist'), he answered as follows: "*Do not imitate the great masters of the past. Seek after the goals which the great men of old pursued*". This is an English translation by Bennett of the original saying in Japanese, "*Kojin no ato wo motomezu, while Kojin no motometaru tokoro wo motomeyo*". With this review, we urge young scientists to seek the goals of the TEM pioneers as well as this great poet, who must have overcome many impediments to prepare the eventual path of research, and who showed us how diligence, tenaciousness, and resilience were able to lead to success. We should also notice that those early TEM pioneers were still young, in their mid-30s to early 40s at the days.

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KONDO, H. & HIPKAEO, W. Lecciones de la literatura de destacados pioneros en los primeros días del análisis microscópico electrónico de células (1940s-1950s). *Int. J. Morphol., 43(1)*:54-69, 2025.

RESUMEN: Se ofrece una descripción general de la historia de la microscopía electrónica de transmisión (MET) con un enfoque en varias preguntas: ¿cuáles fueron las principales formas en que la MET mejoró nuestro conocimiento de la estructura celular más allá de lo obtenido en la microscopía óptica (MO); ¿cómo se revelaron en la MET estructuras intracelulares no reconocidas en la MO?; ¿cómo se estableció la realidad de las estructuras intracelulares observadas recientemente? y ¿qué importancia tuvo la información de referencia acumulada durante la larga historia de la LM para preparar a los científicos que usan la MET para la tarea de encontrar nuevas estructuras intracelulares?. Se espera que esta revisión incentive a los jóvenes investigadores a descubrir detalles de la morfología de las células y los tejidos en un futuro cercano.

PALABRAS CLAVE: Microscopía electrónica; Primeros días de la bio-EM; Hialoplasma; Microscopía óptica; ¿Realidad o artefactos?

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Corresponding author
Wiphawi Hipkaeo
Electron Microscopy Unit, Division of Anatomy
Faculty of Medicine
Khon Kaen University
Khon Kaen 4000
THAILAND

E-mail: wiphawi@kku.ac.th