Ultrastructural histochemistry in biomedical research: Alive and kicking

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Abstract

The high-resolution images provided by the electron microscopy has constituted a limitless source of information in any research field of life and materials science since the early Thirties of the last century. Browsing the scientific literature, electron microscopy was especially popular from the 1970's to 80's, whereas during the 90's, with the advent of innovative molecular techniques, electron microscopy seemed to be downgraded to a subordinate role, as a merely descriptive technique. Ultrastructural histochemistry was crucial to promote the *Renaissance* of electron microscopy, when it became evident that a precise localization of molecules in the biological environment was necessary to fully understand their functional role. Nowadays, electron microscopy is still irreplaceable for ultrastructural morphology in basic and applied biomedical research, while the application of correlative light and electron microscopy and of refined ultrastructural histochemical techniques gives electron microscopy a central role in functional cell and tissue biology, as a really unique tool for high-resolution molecular biology *in situ*.

Introduction

Since the early Thirties of the last century, when the first electron microscope was built, the high-resolution images provided by this revolutionary instrument has constituted an inexhaustible source of information in any research field of both life science[1] and materials science.[2]

The diffusion of commercial transmission electron microscopes and, afterward, of the scanning electron microscopes resulted, from the Fifties to the Seventies, in an extraordinary increase of studies aimed at describing the fine morphology of living or material structures, leading to a remarkable advancement of knowledge. At the same time, histochemical and immunohistochemical techniques, until then prerogative of light microscopy, were applied to electron microscopy, thus allowing the precise location of molecules in cell and tissue components by using electrondense markers.[3-10]

In the field of life science, since 1956 about 270,000 articles (96,000 on biomedical subjects) have been published where electron microscopy was used (source: Scopus database); out of them, more than 47,000 articles or reviews also contained histochemical data (about 44,000 on biomedicine, while 3000 only fall into the field of animal or plant biology). Looking at the distribution of published papers in this timespan (Figure 1), it is evident that electron microscopy was especially popular from the 1970's to 1980's, whereas during the Nineties, with the advent of innovative molecular techniques, electron microscopy seemed to be downgraded to a subordinate role as a merely descriptive technique, with a concomitant decrease in the number of published papers. However, when it became evident that a precise localization of molecules in the biological environment was necessary to understand their functional role, a sort of *Renaissance* of ultrastructural histochemistry took place,[11-28] which has still been continuing during the last decade.[29-41] In this regard, it is interesting to observe (Figure 1) that the articles containing histochemical and ultrastructural data became relatively frequent since the second half of the 1970's, progressively increased in the 1980's, to remain almost constant in their yearly number until now.

May electron microscopy still make a relevant contribution to life sciences?

In recent years, with the advent of the fluorescence super-resolution microscopy, the limit of optical resolution of light microscopy (about 250 nm) was decreased to the 20-50 nm range,[42-44] which enabled to examine cellular details at the nanoscale level, previously unattainable with light microscopes, and approaching the resolution of electron microscopy.[45] In some authors' opinion, super-resolution microscopy "has the potential to replace conventional light microscopy in subcellular imaging questions as the dominant go-to technique",[46] enjoying the benefit of the wide variety of available multicolor histochemical techniques. This even makes it questionable whether transmission electron microscopy (TEM) and scanning electron microscopy (SEM) may still make a relevant contribution to the studies in life science, especially in advanced research fields.

Electron microscopy techniques have been used for a variety of investigations in life science, and it would be very difficult to analyze in detail a so large number of papers in the scientific literature, attempting to understand how electron microscopy was applied, and whether new fields of research may have born, especially in recent years. Thus, I decided to limit my survey to the articles published in the *European Journal of Histochemistry*, taken as an example of a purely histochemical journal that has traditionally been devoted to a wide assortment of subjects in biology and medicine, from functional cell and tissue biology in animals and plants, to cell differentiation and development, to the cellular basis of diseases.

Taking into account the articles published herein during the last decade, it is evident that many studies (on average 12%, with an increase of about 4% in the last three years) either combined histochemical techniques at light microscopy with the high-resolution morphology provided by electron microscopy, or directly applied ultrastructural histochemistry.

The ultrastructural morphological approach at TEM has been widely used in basic and applied biomedical research for the study of different tissues under unperturbed or experimental conditions: tooth structure and repair;[47,48] white adipose tissue classification;[49,50] stem cells culture for reconstructive purpose;[51,52] skeletal muscle features under experimental[53,54] and pathological[55,56] conditions; liver response to pretransplantation treatment;[57] female reproductive organs[58] and explanted oocytes for *in vitro* reproduction;[59,60] post-implant skin modification;[61] autopsy myocardium for diagnostic purposes[62].

Fine morphology at TEM has also been applied in cell[63-67] and developmental biology,[68-70] and was essential to describe the fine morphology of tissue and organs of differ-

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Key words: Transmission electron microscopy; scanning electron microscopy; high resolution.

Received for publication: 25 October 2018.
Accepted for publication: 31 October 2018.

European Journal of Histochemistry 2018; 62:2990
doi:10.4081/ejh.2018.2990

ent animal species.[71-74] In recent years, morphology at TEM proved to be crucial in nanomedicine to describe the interactions of nanoconstructs with different cell components.[38,75] Finally, morphological analysis at TEM has been applied to reveal the structural preservation of explanted organs or tissues maintained *in vitro* in innovative fluidic systems.[76] The three-dimensional ultrastructural morphology provided by SEM contributed to the detailed characterization of bone[77] and adipose tissue.[49,50,78]

Ultrastructural morphological data have been combined to Energy Dispersive X-ray (EDX) microanalysis in biomedical research and diagnosis[79] to detect asbestos fibers and metal contaminants in lung carcinomas,[80,81] or to evaluate the biocompatibility of bone cements for reconstructive purposes,[82] as well as to describe the effect of pollution on marine organisms in environmental research.[83]

Recently, TEM and atomic force microscopy have been used in a correlative approach, to characterize the byssus threads of *Pinna nobilis*[84] or the protein globoids and starchy granules in the seeds of different cereals.[85]

Besides the use of electron microscopy as a high-resolution morphological support to light microscopy histochemistry, many authors directly applied ultrastructural cytochemistry and immunocytochemistry to various research fields.

A cytochemical approach using osmium ammine staining allowed to describe the DNA organization in the chromatin structure of mammalian nuclei.[86] In several papers, diaminobenzidine photo-oxidation was applied to visualize fluorescent probes at TEM: by this approach, calcium ions were detected and located in the endoplasmic reticulum after staining with Mag-Fura 2 dye,[87] the uptake and intracellular fate of different fluorescently-labelled nanoparticles was monitored,[88,89] and the different subcellular compartments involved in the endocytosis routes were precisely described after labelling the plasma membrane with the fluorescent dye, PKH26.[90] The possibility to combine diaminobenzidine photo-oxidation and gold immunolabelling was also demonstrated.[91]

Ultrastructural immunocytochemistry has largely been used to visualize specific proteins in cultured cells,[66,92-94] as well as in calcified tissues,[47,68,95] in the nervous tissue,[96,97] in the skeletal muscle,[56,98] and in the gonads.[99] Immunocytochemistry at TEM has been coupled to Field Emission in Lens Scanning Electron Microscope,[100-102] and has been performed in samples prepared for SEM, too.[103]

Concluding remarks

It is therefore clear that electron microscopy not only has maintained its fundamental role in histochemical studies in a variety of, let's say, *traditional* research fields (cell and developmental biology, biomedicine, zoology), but has likewise proven to be useful in novel research areas such as nanotechnology and regenerative medicine. Moreover, the successful association of the ultrastructural approach with other powerful high-resolution techniques (*e.g.*, X-ray microanalysis and atomic force microscopy) demonstrates the great versatility of electron microscopy, thus accounting for the increase of its utilization by scientists in recent years.

As much as super-resolution light microscopy, electron microscopy requires expensive equipment, highly qualified personnel and time-consuming protocols, which are all detrimental characteristics in the present research word ruled by the "publish or perish" imperative; despite this limit, these techniques are essential for biomedical research where the detection of single molecules needs to be associated to their precise location, at the subcellular (or even sub-organellar) level.[10] Actually, to mechanistically understand the function of an organelle or a macromolecular complex, the composition and structure of its molecular components must be viewed in the frame of their spatial organization within the cell; thus, imaging molecules will continue to remain a crucial issue in biomedical research, in the years to come.

Electron microscopy will continue to be irreplaceable for ultrastructural morphology in basic and applied biomedical research: it still has better resolution than fluorescence super-resolution microscopy, and has the advantage to allow a direct visualization of both the membrane-bounded and cytosolic structural components of the cell, whereas in super-resolution microscopy all these structures are indirectly resolved through the labelling of their molecular components by fluorescent probes.

Correlative light and electron microscopy (CLEM) methods effectively integrate the advantages offered by fluorescence microscopy and electron microscopy:[105-107] in fact, while light microscopy allows to screen relatively wide areas of the sample where multiple molecular species (proteins, carbohydrates, lipids and nucleic acids) may simultaneously be detected by specific labelling, electron microscopy makes it possible to spatially visualize both labeled and unlabeled structures at the highest resolution.

Nowadays, the application of CLEM and of refined ultrastructural histochemical techniques is giving back electron microscopy its central role in functional cell and tissue biology, as a really unique tool for high-resolution molecular biology *in situ*.[108]

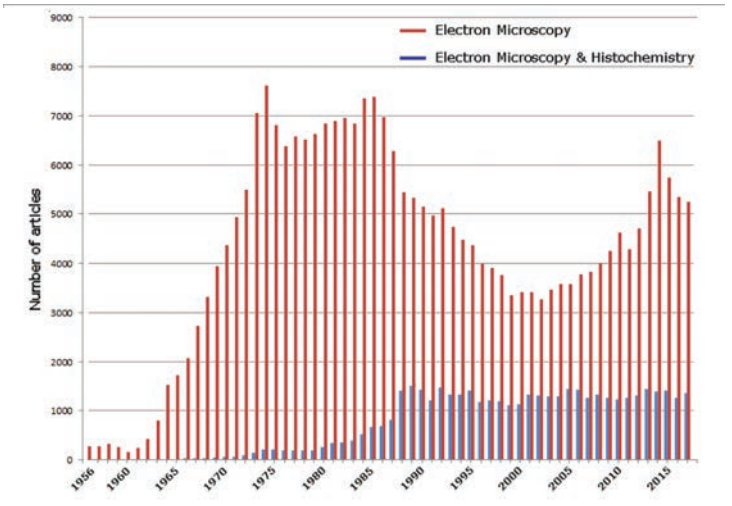

Figure 1. Number of scientific articles containing electron microscopy data published since 1956.

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