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Electron Microscopy Now-a-Days

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Introduction

Studying the structural and functional properties of biomolecules requires to develop experimental paradigms not only to recognize but also to locate and track these molecules within bodies, organs, tissues, cells and/or subcellular compartments. The biologist is faced with a wide range of observation techniques which are continuously evolving. But how to determine from the element to analyses which technology(s) is the most appropriate? A rigorous repertory of questions needs to be asked: what is the size of the biomolecule under study? Is it outside or inside the cell? Nuclear or cytoplasmic? Answering these questions are the first steps towards elucidating its function.

Thus, the questions of the biologist are fundamental, and a relevant strategy is decisive insofar as each technology requires a significant investment. Moreover, each technical approach has its own strengths and limitations and usually only gives a partial answer. The choice becomes more and more difficult as new approaches continuously open up very innovative and fruitful fields of investigation. Should we favor a new technology with its new openings without neglecting the traditional techniques that have been the substance of many discoveries in the past?

We will discuss these questions by taking a few examples of microscopes. First, we will distinguish microscopies from the point of view of their magnification and their resolution power for structural and sub-structural analysis. Then, we will discuss how microscopy can allow the visualization and location of a biological object under the closest conditions of life. The magnification of a microscope corresponds to the ratio between the apparent diameter of the image and the apparent diameter of the object seen with a naked eye at the minimum distance of distinct vision. The resolution power of a microscope, which sets the limits in the size of the observable details, is related to the wave character of the light. Thus, the smallest "discernable" detail of the sample is correlated to the wavelength and the numerical aperture of the lens.

Optical Microscopy

In optical microscopy, the magnification can reach 1000 X and the resolution is about 200 nm. It can approach 160 to 180 nanometers using confocal microscopy. For structural observation, the samples are fixed, treated then embedded in

paraffin. Observations of tissues or cells on stained sections are easy as this technique is commonly used. In electron microscopy, the magnification theoretically reaches 100X million but as lenses are often not perfect, magnification is in practice 1X million. Instead of using photons as in optical microscopy, these microscopes use a beam of accelerated electrons, which are projected onto the sample using electrostatic and electromagnetic lenses. As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, transmission electron microscopes can achieve a resolution of 0.1 nm. With such technical parameters, electron microscopes are usually used to investigate the ultrastructure of a wide range of biological and inorganic specimens. In these cases, conventional electron microscopy by transmission (MET) is the best choice. As the electron flow launched under vacuum can damage the samples, they are previously fixed and dehydrated before being embedded in epoxy resin. Sectioning permits to obtain 50-70 nm sections. The slides are next contrasted using uranium and lead salts allowing ultrastructural analysis of the biological samples. The observations are revealed by "grey scale" and a good knowledge of the specific "grammar and dictionaries" of MET is needed to make observations easier. Modern MET produces electron micrographs using specialized digital cameras and frame grabbers to capture images (**Figure 1**).

Detection of specific biological elements at cellular and ultracellular levels requires further considerations. In order to highlight specific biological element inside or outside the cell, immunofluorescence is a widely used technique. The molecule to study must be labelled with a fluorochrome which is a chemical substance that emits light after being excited with a light of a specific wavelength. The emitted photon has a longer wavelength than the exciting photon. Fluorescence can be obtained in a number of ways: genetically encoded substances, such as the green fluorescent protein (GFP), emit visible fluorescence and are used to study dynamic processes in living cells. However, for non-fluorescent substances, a fluorochrome must be tagged to an antibody or the element itself (DAPI for DNA, rhodamine or fluorescein for protein).

The microscope must have a defined range of coherent wavelengths or lasers with distinct wavelengths for the fluorescent object to be excited and subsequently detected:

- Confocal microscope offers several advantages over conventional widefield optical microscope. Indeed, the presence of a confocal pinhole allows to reject out of focus fluorescent light. By scanning many thin sections through the sample (optical sections), it is possible to build up a very clean three-dimensional image of the sample.
- Pulsed laser photonic microscope combines the qualities of the confocal microscope with two-photon excitation in long wavelengths of low energy. These characteristics are well-suited to image living cells as they cause less damage than short-wavelength lasers typically used for single-photon excitation.

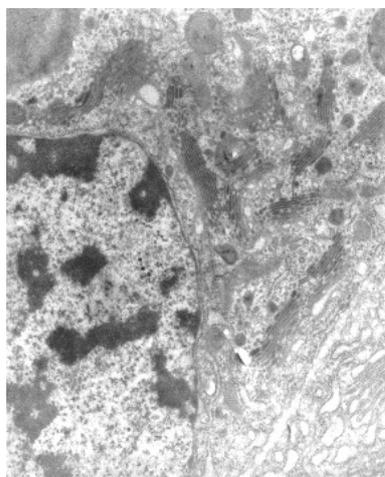


Figure 1 Ultrastructural analysis of subcellular structures of a cell (Transmission Electron Microscopy) the nucleus contains euchromatin and heterochromatin, the nuclear envelope is in relation with a well-developed ergastoplasm, the golgi apparatus produces secretion globules in its neighborhood. All these observations are in favor of a cell in intense activity.

Comparison		Optical Microscopy	Electron Microscopy (MET)
Parameters of the Microscope	Magnification	1000x	1000,000x
	Resolution power	160-180 nanometres	0.1 nanometre
Tissue condition	Native state	+	+ Cryo-electron Microscopy Vitrification
Tissue observation	Overview of the tissue	+++	+
	Cellular observation	.. +	++
	Subcellular observation	.. +	+++
Detection of specific biochemical elements inside the cell		Fluorescence	Colloidal Gold 5-10 nanometres

Jacques Dubochet, Joachim Franck and Richard Henderson won the Nobel Prize in Chemistry in 2017 for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution. They made a quantum leap in advancing ultra-structural observations of cells or tissues in their native state unlike conventional preparations. No dyes or chemical fixatives are used, and the samples are rapidly frozen in liquid ethane at very low temperatures. They are thus frozen in their native state in an amorphous ice (vitrification). Subsequent image processing allows sample registration, contrast enhancement and 3-dimensional image reconstruction.

Using electron microscopy, it is now possible not only to detect biochemical elements at the subcellular level, but also to preserve the elements to study under conditions identical to living conditions.

Discussion

All these microscopes make possible the precise localization of the element of interest on cells or tissues, fixed or alive. The confocal microscope and the biphoton greatly improve the quality of observation as well as the preservation of the sample. Nevertheless, the resolution is always limited by the wavelengths of the light.

This is where electron microscopy is of major interest. With conventional electron microscopy, it is not only possible to access to ultrastructural morphology but also to localize biochemical elements inside the cell using antibodies labelled with colloidal gold particles of 5-10 nanometers. However, this technique also has its own limitations. Indeed, the samples need to be processed in such a manner (**Table 1**) that no observation can be done on living samples.

Table 1 Comparison of optical microscopy with electron microscopy.

Conclusion

Each microscopy technique is valuable but limited: fluorescent and confocal microscopes, associated with live cell imaging are often used to ascertain the position of a fluorescently tagged small molecule. However, the resolution of very small structures is limited.

On the other hand, electron microscopy is able to reveal details of subcellular morphology at very high resolution. Combined with cryo-electron microscopy, the biological elements are observed in conditions approaching life conditions. However, its high resolution impedes to get an overview of the biological structure, making it difficult to measure highly dynamic processes with precision.

Is it possible or necessary to combine several techniques to address precisely biological questions? Increasing laboratories have been focusing on correlative microscopy. The purpose is to fill the gap between light microscopy and EM (CLEM) at the level of the biological sample. This purpose needs some

technical prowess (sample processing, precision of benchmarks, development of associated image treatment) and coherent technological platforms. However, the high quality confocal or bifocal microscopies, the improvement of cryo-electron microscopy have opened new avenues in this field. In

fine, it will be suitable to observe the same biological sample both at optical and electronic levels using a single microscope able to work both with light and electron. This goal is partially achieved. In this way, electron microscope still has a promising future.