
# IYSC

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## WORKING GUIDE ON BIOMEDICINE AND IMAGING

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## Introduction to microscopy

Since the beginning of humankind man has been enquiring about his surroundings and about himself and his own nature. The need for answers has resulted in the need for more and more knowledge. It was at the end of the XVI century and during the XVII century when there was a Scientific revolution that established a method based on observation, question making and experimentation. The first scientific societies appeared and the journals started publishing their research.

It is actually during this period of time when we can trace the origins of the first microscope with Janssen father and son and Galilee Galilei. The first instruments consisted on two lenses placed at both ends of a rigid cylinder. These instruments were inaccurate and had little resolution. Naturalist Leeuwenhoek, with his ability to polish lenses managed to advance in microscopy. From that moment there was a new field for mankind, the discovery of the microscopic world, the new forms of life, the concept of cell, the use of this instrument was unstoppable. Improvements of the optic microscope occurred in the 19th century when the chromatic aberrations of lenses were corrected and the use of immersion objectives allowed maximum optical resolution. From that moment advances regarding the optic microscope have been few like the fluorescent microscope, the phase-contrast microscope or the confocal microscope.

At the beginning of the 20th century Hans Busch developed the first magnetic lenses which were capable of directing an electron beam in a similar way to how the optical lens does it with light. A few years later, in 1931, physicist Ernst Ruska, together with engineer Max Knoll, used that discovery to build the first Transmission Electron Microscope (TEM). It was an instrument which was capable of improving the likely resolution of the optical microscope thanks to a lower length wave of electrons regarding the visible light photons. In the following years, Ernst together with Bodo von Borries worked on improving the electron microscope while his brother, Medicine PhD Helmet Ruska, started to use these microscopes for medicine and biology research. He was the first one to study the structures of diverse microorganisms, especially viruses, totally unseen under the optical microscope. In 1937, Manfred von Ardenne developed the first Scanning Electron Microscope (SEM). While TEM uses the electrons transmitted through the sample in order to show a projected image of it, SEM uses an electron beam to scan a sample covered by carbon or a metal in order to obtain a 3-D image of the surface.

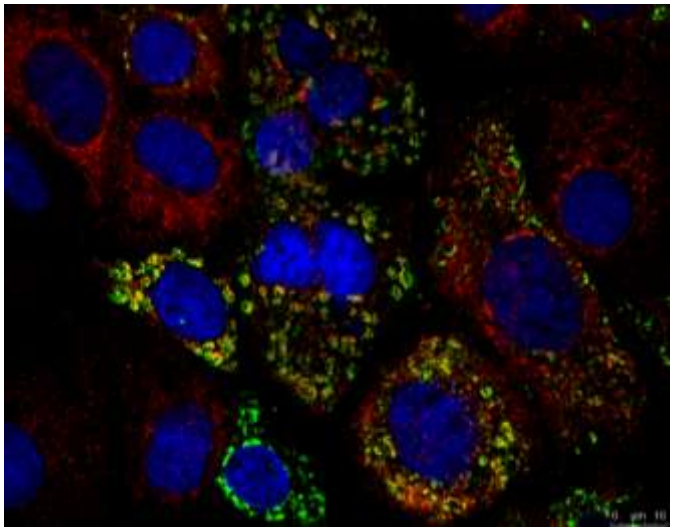
## Optical microscopy of biological samples

Direct observation of biological specimens through an optical microscope is a challenge due to the fact that the samples and the medium where they lay are practically the same: water. The slight difference in their diffraction indexes result in little contrast, making differentiation of the sample and the medium quite difficult .
In order to observe the samples it is necessary to improve the contrast so that they can be distinguished from the medium. One of the most common ways is using specific colouration, through staining with one only stain (monochromatic staining) or using a combination of two or more stains. The type of microscope used for the observation of the samples treated that way is called bright-field microscope. Observation is simplest because contrast between the medium and the sample is highly evident. These methods are commonly used in parasitology, bacteriology, histology, etc
The use of stains involves, in most cases, the death of the sample under observation because in order to preserve it, the sample requires a fixation process. Nevertheless there are circumstances where the viability of the samples needs to be preserved and we need to use other techniques that don't imply a fixation process or the use of stains. It is noteworthy the case of cell cultures, widely used both in diagnose and in research. Every culture room has a microscope to observe the state of the culture, its growth, its cytopathic state, etc. In these cases it is used an inverted microscope equipped with a

system that uses the properties of light (phases differences) in order to highlight and make it possible to see the outline of the cells on the medium where they are growing in. This microscope is called phase-contrast microscope and has two-phase rings that provide the necessary contrast. Another microscope that benefits from the properties of light (interference difference) in order to highlight the sample over the medium without the need of stains is the differential interference contrast (DIC) or Nomarsky. This microscope uses a series of polarizers and prisms to observe the relief of the sample and its 3-D perception.

Another technique to observe both living and fixated samples is fluorescence microscopy based on the capacity of certain substances to absorb energy from a certain wider wave length. The fluorescence microscope started being used in the first decade of the 70's in the XX century and it has evolved in the last years into the confocal microscope. It's is a highly-sophisticated fluorescence microscope with high quality and high resolution, capable of eliminating the out-of-focus light, allowing more optical sections of the thick samples.

Fluorescence requires simplest methods that use stains which have affinity for a certain component of the cell. The most common method is the cell nucleus staining through the use of DNA-binding intercalators, like DAPI, but there are many more, like Mito Tracker, which stains mitochondria, phalloidin, used to mark the cytoskeleton of actin, membrane dying, lipids and a lot more. This type of staining is usually combined with more complex processes like immunofluorescene, where specific antibodies are used together with fluourochromes to detect certain proteins. This technique is highly common both in research and disease diagnose. Another type is the use of a nucleic-acid probe fluorescence-labeled. This technique is knows as fluorescence in-situ hybridization (FISH), highly applied in the diagnosis of genetic diseases.



Culture of MA104 cells infected by Rotavirus. After 6 hours of the infection, the culture was fixated and labeled with DAPI stain, which dyes the nucleus blue, and a double immunofluorescene in red to detect the endoplasmic reticulum and green to detect viral protein NSP4. The yellow and orange areas show where there is a colocalization between the viral protein and the reticulum

Leaving aside the techniques of light-transmitted microscopy (Bright field, phase contrast and interdifference contrast) and focusing on fluorescence techniques (epifluorescence and confocal), we can consider that these techniques are used in two major fields; on the one hand, in biomedical research and, on the other, in clinical diagnosis.
Fluorescence microscopy used in diagnostic microbiology is associated to the use of immunofluorescence techniques to detect direct presence of the pathogen or as evidence of its presence thorough detection of specific antigens. Diagnostic laboratories are usually equipped with simple epifluorescence microscopes to observe the samples and show negative or positive depending on the result of the observation and the controls carried out. This diagnostic technique is usually associated to other molecular techniques like PCR or ELISA

On the other hand, epifluorescence microscopes are common in research labs of certain areas, like cell biology, microbiology, etc. They are also equipped with computers and highly-sensitivity cameras capable of capturing fluorescence. For highly-accurate colocalization studies among proteins or 3-D capture of images we use a more sophisticated microscope, the confocal microscope, which allows great accuracy of optical sections of the samples under observation, allowing one only plane regardless the thickness of the sample. It also allows reconstruction of the optical sections taken to obtain a 3-D image of the preparation.

**Electron microscopy of biological samples**
Despite the benefit of its greater resolution, the electron microscope, compared with the optical microscope, is limited, at a certain degree, concerning biological material observation. The electron beam strongly interacts with matter, which implies that the electron microscope column should be in high vacuum (the samples should undergo a dehydration process) and also the fact that the electron beam should damage the sensitive biological samples. Besides this, electrons have a limited penetration capacity (usually 0.5-1 μm). All these limitations together make it necessary to prepare the specimens under study in certain ways, depending on the nature or the types of structures to be analysed.

In the case of macromolecular solutions or small microorganisms (smaller that the penetration capacity of the electrons), their observation is possible using negative staining techniques and electron cryomacroscopy. In the negative staining technique, the specimen under study is absorbed on a carbon surface previously ionized and later embedded in a metal salt of a heavy electron dense element, resistant to electron damage (uranyl acetate, uranyl formate, phosphotungstate ammonium, silicotungstate ammonium, etc.)

This process, on the one hand, preserves the sample and offers its inverted or negative image; because where there is biological material (lower electron dense) electrons are transmitted and where there is a heavy-metal salt (higher electron dense) the electrons are stopped. The main advantages of these techniques are the simple process of preparation and the high contrast of the images obtained. On the other hand, the need to adhere the sample to a surface, the dehydration intrinsic to the technique and the use of staining agents may alter the basic structure under study

In the case of electron cryomicroscopy the sample is frozen at a temperature close to liquid nitrogen at a high speed in such a way that water acquires a vitreous and amorphous structure. Unlike "conventional" ice, with a crystalline structure, vitreous ice has no structure and is lower electron dense. Using this technique it is possible to observe directly the macromolecules in their native and hydrated state, because the low temperatures protect the specimen from dehydration and, partially, from electron damage. However, the slight difference in electron density between vitreous ice and the biological material that it embeds makes contrast quite low. Besides, it is possible to obtain information on the surface of the sample by generation of replicas and metal shading of it. Finally, using modern techniques of digital image processing it is possible to recover 3-D information of the structures under study out of the projected images obtained through negative staining or electron cryomicroscopy

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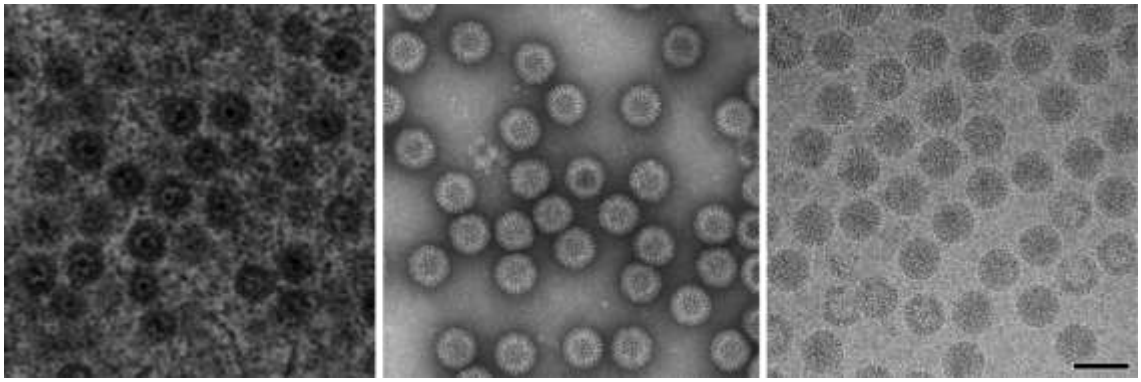

Rotavirus visualized under electron microscopy through analysis of ultra-thin sections (left), negative staining (center) and electron cryomicroscopy (right). Scale bar: 100 nm.
In the case of bigger samples like cells, tissues and organs, the electron beam is unable to pass through the specimen; therefore it is necessary to obtain ultra-thin sections in order to observe the ultrastructure of the sample under study.
During this process the sample must be fixated using chemicals like formaldehyde, glutaraldehyde and osmium tetroxide; dehydrated and included in plastic paraffin. Later, the sample should be cut using an ultratome to obtain very thin sections (normally less than 100nm) allowing electrons passing through.
Such sections are contrasted through solutions of heavy metals like lead citrate and uranyl acetate to be later observed under electron microscopy. If the dehydration and inclusion processes undergo low temperatures, it is called cryosubstitution and it results in better preservation of the structure under study. On the other hand, like in smaller specimens, it is possible to generate metal replicas that give topological or surface information of the sample. Finally in a similar way to immunofluorescene in optical microscopy, through electron immunomicroscopy techniques, it is possible to locate molecules by using specific antibodies coupled to colloidal gold (electron dense).
Since its beginnings, microscopy has had a fundamental role in modern cell and structural biology. Currently is is key to characterization of molecular mechanisms underlying disease (both diseases caused by microorganisms and non-infectious diseases), and to determine cell and tissue ultrastructure, structure of microorganisms and macromolecular complexes in their natural contest, etc.

Microscopy has also been key in the field of virology, including the discovery of a large number of viruses, viral diagnosis and research of virus-cell interaction

Although in the field of diagnosis, electron microscopy has been replaced by modern molecular techniques like polymerase chained reaction or PCR, these techniques are only applied to well-known and characterized pathogens. This fact together with its swiftness and potential capacity to visualize practically any virus make electron microscopy an essential tool to detect and characterize emergent infections of sanitary importance, new pathogenic agents and bioterrorist alerts.

In the case of structural biology, the combination of modern 3-D techniques, like single-particle analysis and electron tomography, allows visualization of the architecture of great complexes, reaching an atomic or quasi atomic resolution. In the field of cell biology,

modern techniques of sample preparation, like high-pressure freezing and cryosubstitution, allow greater preservation of the cell structures under study. This case together with the improvement of more powerful microscopes are allowing characterization of sub-cellular structures in their native hydrated environment at the same time such structures and contextual used spatially.

**Potential topics for discussion**

- As we have seen, there is a great variety of techniques for optical microscopy, but one of them is essential in every laboratory that works with cell cultures. What type of microscope is used in these type of labs? Why do you think so? Which are its physics fundamentals?
- In many hospitals and diagnostic laboratories simple microscopes are used to observe histological samples, they are bright-field microscopes, however, the process of "preparation' of the sample can be hard, what methods to prepare a sample can be applied to carry out this diagnosis? Which are most common staining techniques?
- Fluorescence microscopy is a type of optical microscopy applied to diagnosis and research. Which are the principles underlying? And which are the specific components of these microscopes?
- Usually fluorescence microscopy is associated to the use of specific antibodies through the immunofluorescene techniques. Which are the principles of this technique? Which results can be obtained?
- A more sophisticated fluorescence microscope is the confocal microscope, which are its fundamental principles? Which are, in your opinion, the components that are different in other microscopes?
- With the confocal microscope we have reached the theoretical maximum optical resolution. This threshold can be improved with the FRET technique, with the super-resolution microscopes and with electron microscopes. What is the FRET technique?
- Super-resolution microscopes have meant a historical milestone, why? How do they work?
- Which are the applications of electron microscopy in pathogen diagnostics? What pathogens have been discovered thanks to electron microscopy? Which pathogens are diagnosed through electron microscopy
- Which is the role of microscopy in emergent diseases and bioterrorist alerts?
- High-resolution electron microscopy: is it possible to determine the structure of a macromolecular complex at an atomic level through microscopy? Where are the boundaries?
- New microscopy: Which new microscopy is developing?

**Information Sources**

- The source for microscopy education (Nikon).
  https://www.microscopyu.com/
- Microscopy Resource Center (Olympus).
  http://www.olympusmicro.com/index.html
- Molecular expressions:
  http://micro.magnet.fsu.edu/index.html

- MyScope (Australian Microscopy & Microanalysis Research Facility)
  http://www.ammrf.org.au/myscope
- Introduction to electron microscopy (FEI)
  http://www.fei.com/introduction-to-electron-microscopy/
- Microscopy websites
  https://www.jic.ac.uk/microscopy/links.html
- Blog Ciencias biosanitarias para todos:
  http://gonzalez-camacho.blogspot.com.es/