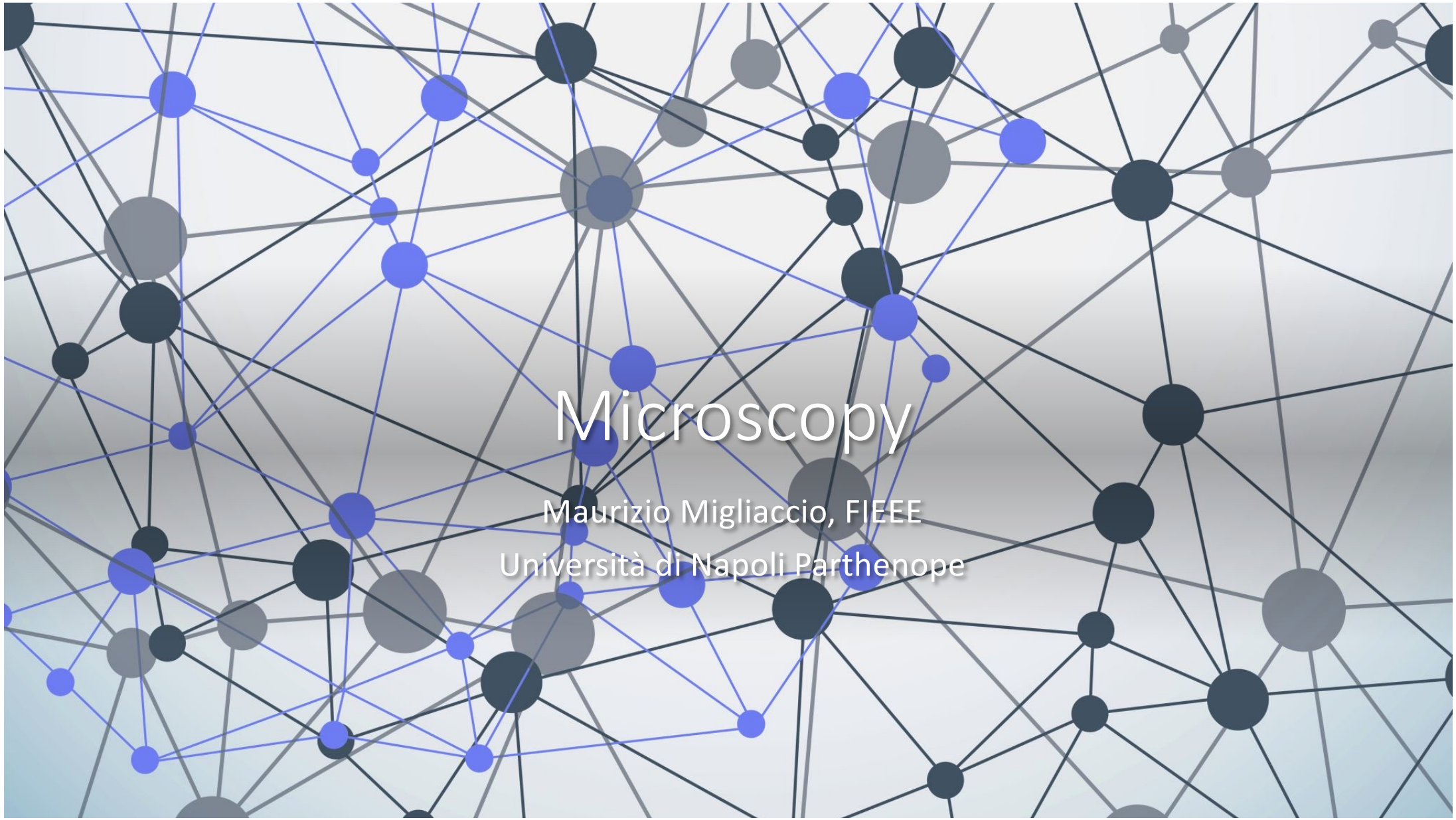


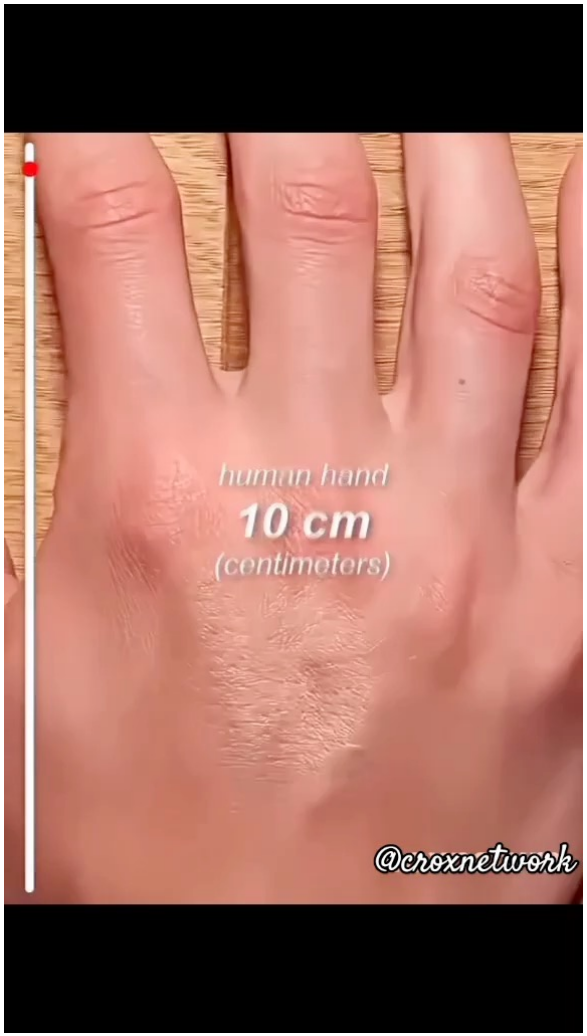
Microscopy

Maurizio Migliaccio, FIEEE
Università di Napoli Parthenope

Quantum dive

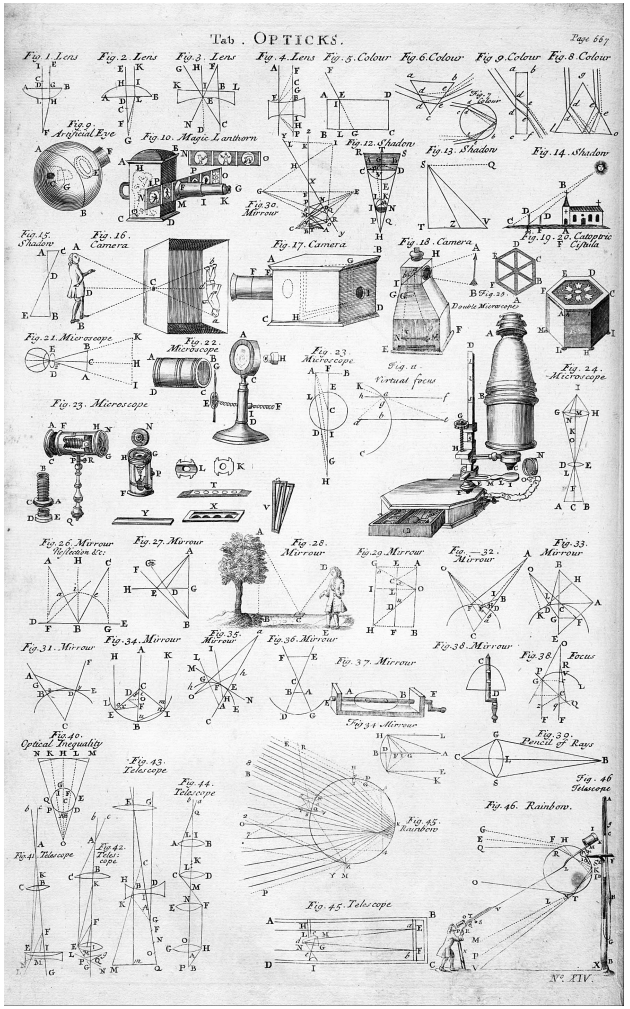
From the Paestum diver to the quantum dive

https://www.youtube.com/watch?v=muUwkheug0E



Optics

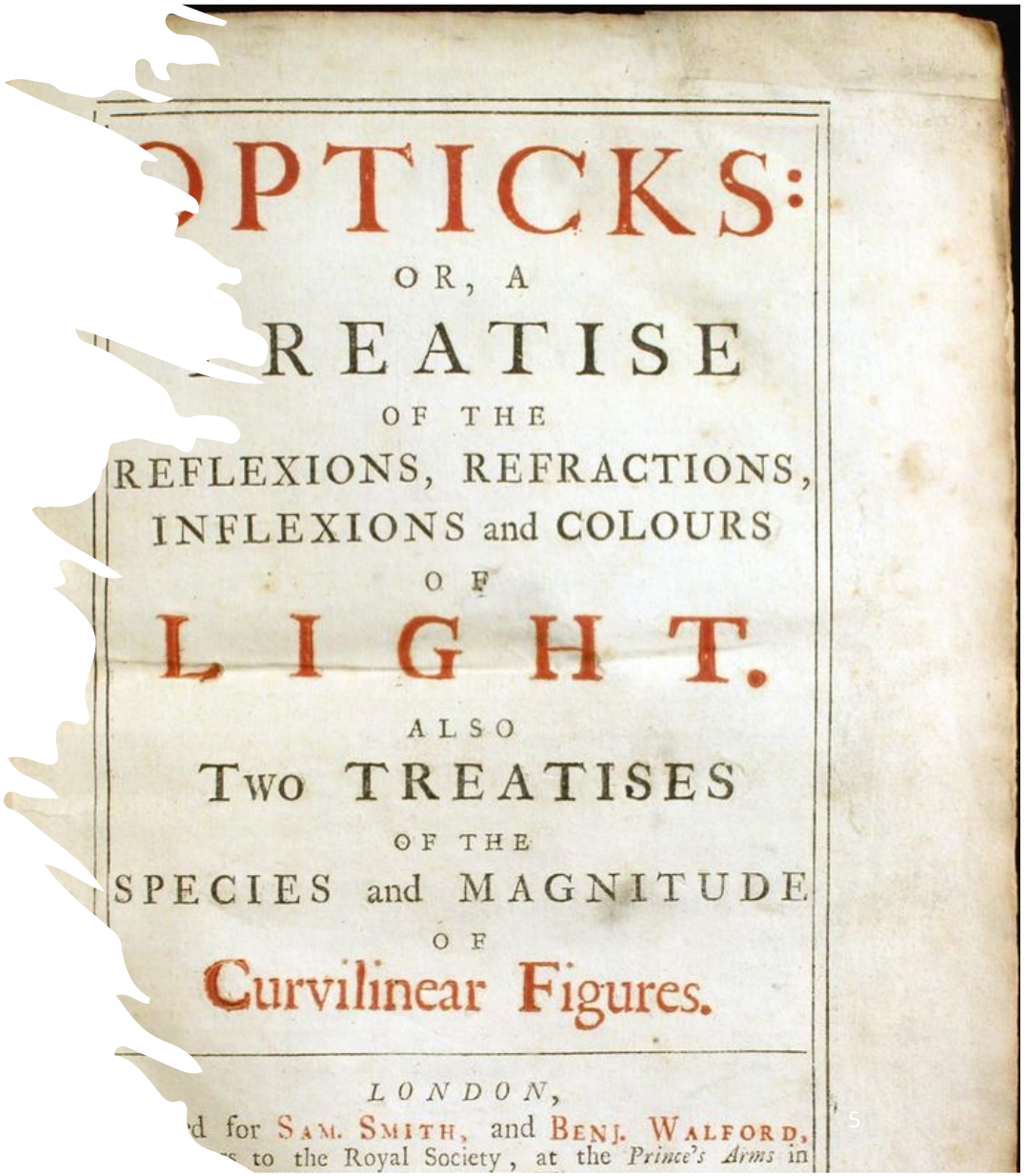
- Optics is the branch of physics that studies the behavior and properties of light, including its interactions with matter and the construction of instruments that use or detect it.
- Optics usually describes the behavior of visible, ultraviolet, and infrared light.
- Aside: *Table of Opticks, 1728 Cyclopaedia*



Isaac Newton

- Isaac Newton (25 December 1642 – 20 March 1727) was an English mathematician, physicist, astron omer, alchemist, theologian, and author who was described in his time as a natural philosopher.
- From 1670 to 1672, Newton lectured on optics.
- In 1704, Newton published *Opticks*, in which he expounded his corpuscular theory of light. He considered light to be made up of extremely subtle corpuscles.

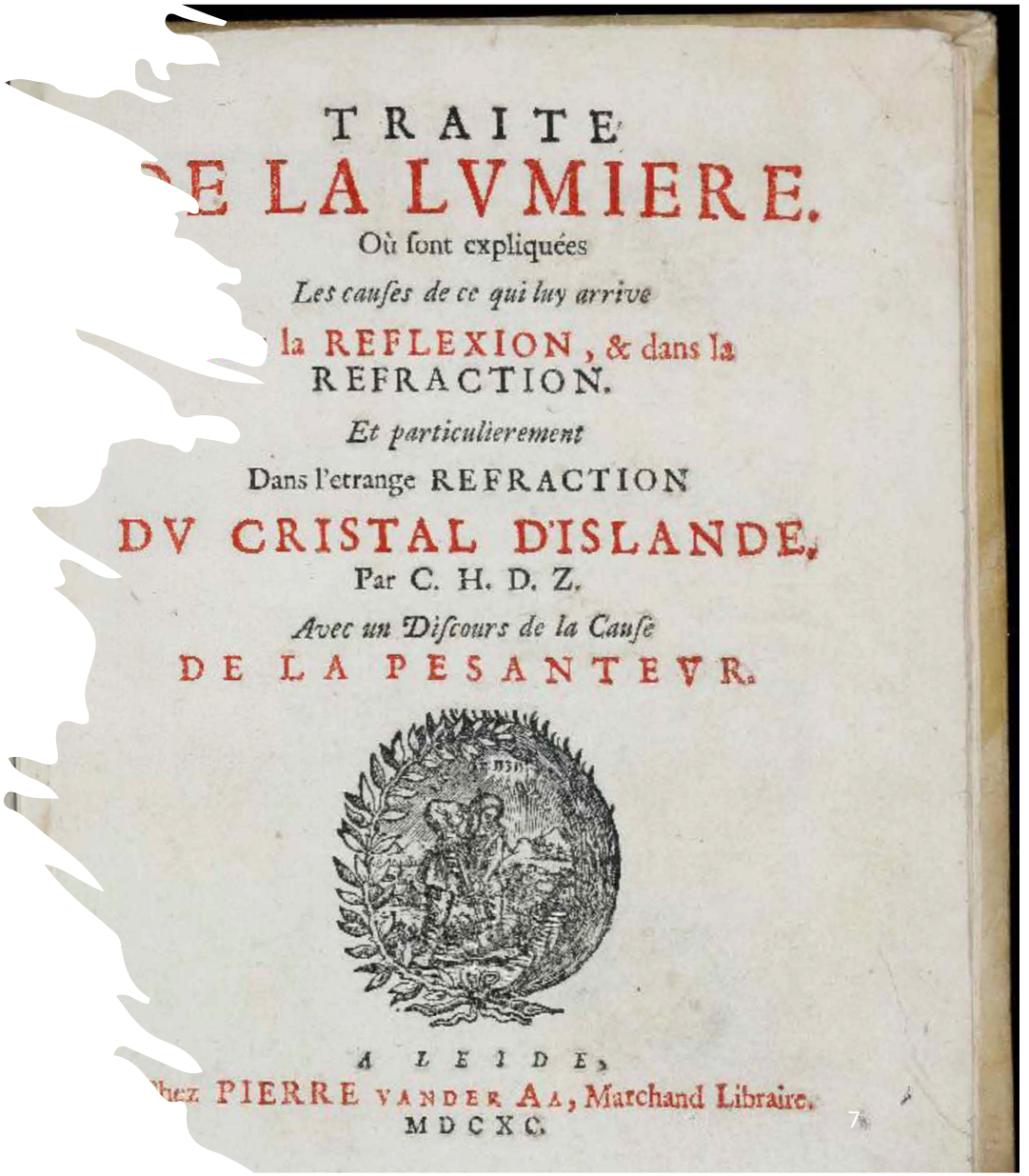
What is light ?



Christiaan Huygens

- Christiaan Huygens, (14 April 1629 – 8 July 1695) was a Dutch mathematician, physicist, engineer, astronomer, and inventor who is regarded as a key figure in the Scientific Revolution.
- In optics, he is best known for his *wave theory of light*, which he described in his Traité de la Lumière (1690).
- His theory of light was initially rejected in favour of Newton's corpuscular theory of light, until Augustin-Jean Fresnel adopted Huygens's principle to give a complete explanation of the rectilinear propagation and diffraction effects of light in 1821. Today this principle is known as the Huygens–Fresnel principle.

What is light ?


TRAITE
DE LA LVMIERE.
Où sont expliquées
Les causes de ce qui luy arrive
dans la REFLEXION, & dans la
REFRACTION.
Et particulierement
Dans l'etrange REFRACTION
DV CRISTAL D'ISLANDE.
Par C. H. D. Z.
Avec un Discours de la Cause
DE LA PESANTEVR.
A LEIDE,
Chez PIERRE VANDER AA, Marchand Libraire.
MDCXC.

Thomas Young

- Thomas Young FRS (13 June 1773 – 10 May 1829) was a British polymath who made notable contributions to the fields of vision, light, solid mechanics, energy, physiology, language, music al harmony, and Egyptology. He was instrumental in the decipherment of Egyptian hieroglyphs, specifically the Rosetta Stone.
- Young has been described as "The Last Man Who Knew Everything". His work influenced that of William Herschel, Hermann von Helmholtz, James Clerk Maxwell, and Albert Einstein.
- Young is credited with establishing Christiaan Huygens' wave theory of light, in contrast to the corpuscular theory of Isaac Newton

Thomas Young

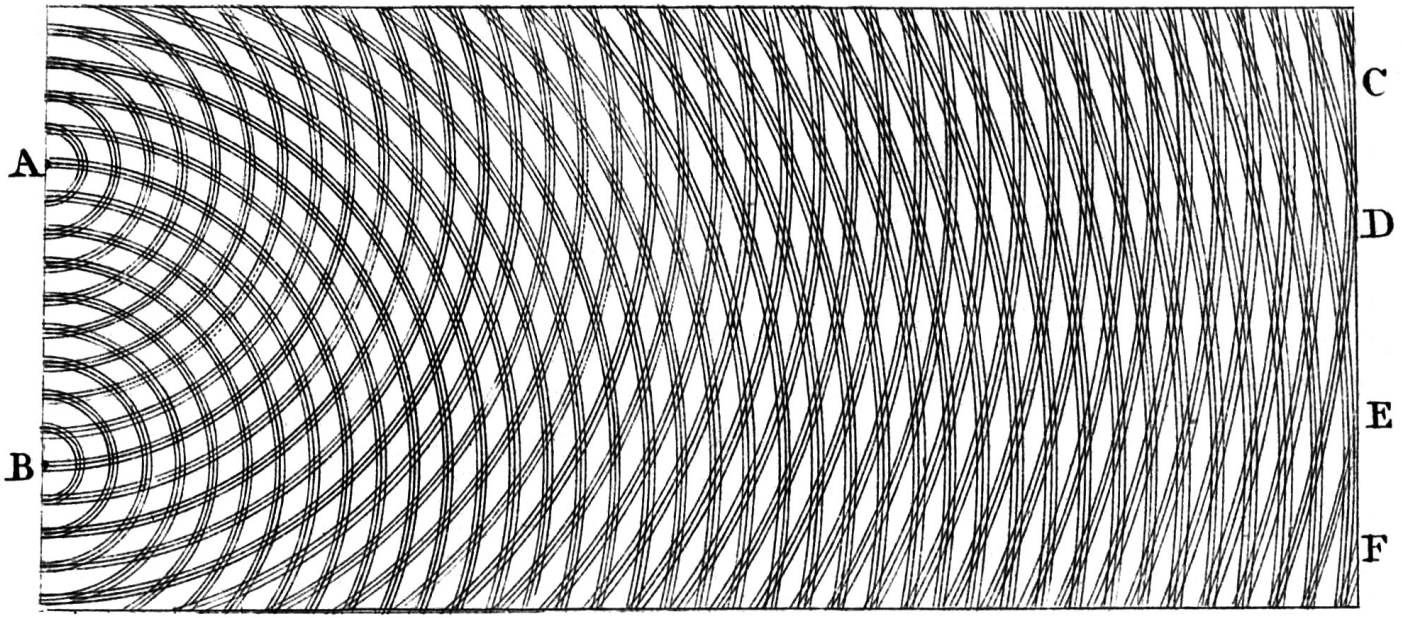
In 1801, Young presented a famous paper to the Royal Society entitled "On the Theory of Light and Colours" which describes various interference phenomena.

In 1803, he described his famous interference experiment.

https://royalsocietypublishing.org/doi/pdf/10.1098/rstl.1802.0004

Thomas Young

- Young's interference experiment, also called Young's double-slit interferometer, was the original version of the modern double-slit experiment, performed at the beginning of the nineteenth century by Thomas Young.
- This experiment played a major role in the general acceptance of the wave theory of light.



Optics

- Classical optics is divided into two main branches: geometrical (or ray) optics and physical (or wave) optics. In geometrical optics, light is considered to travel in straight lines, while in physical optics, light is considered as an electromagnetic wave.
- Geometrical optics can be viewed as an approximation of physical optics that applies when the wavelength of the light used is much smaller than the size of the optical elements in the system being modelled.

Frits Zernike

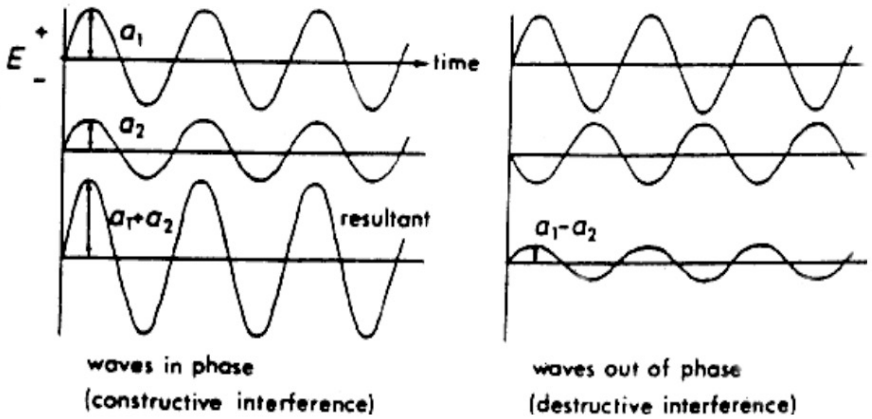
- Frits Zernike (16 July 1888 – 10 March 1966) was a Dutch physicist and winner of the Nobel Prize in Physics in 1953 for his invention of the phase-contrast microscope.

Phase-contrast microscopy

- The phase-contrast microscope has revolutionized biomedical research on living cells, which, not being colored (as it would require the use of fixers and manipulators which can cause morphological and chemical modifications with death of the cells themselves), are practically invisible under the microscope.
- However, the light velocity changes and by means of a device that measures the phase difference between the biological material and a reference one, the biological material can be emphasized.

Phase-contrast microscopy

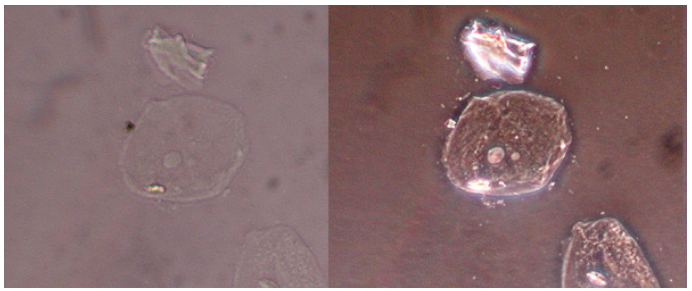
- When light waves travel through a medium other than a vacuum, *interaction with the medium causes the wave amplitude and phase to change in a manner dependent on properties of the medium*.
- Changes in amplitude (brightness) arise from the scattering and absorption of light, which is often wavelength-dependent and may give rise to colors.
- Photographic equipment and the human eye are only sensitive to amplitude variations. Without special arrangements, phase changes are therefore invisible. Yet, phase changes often convey important information.



Phase-contrast microscopy

- Phase-contrast microscopy is particularly important in biology. It reveals many cellular structures that are invisible with a bright-field microscope.
- These structures were made visible to earlier microscopists by staining, but this required additional preparation and death of the cells.
- The phase-contrast microscope made it possible for biologists to study living cells and how they proliferate through cell division.
- It is one of the few methods available to quantify cellular structure and components that does not use fluorescence.

The same cells imaged with traditional bright-field microscopy (left), and with phase-contrast microscopy (right)

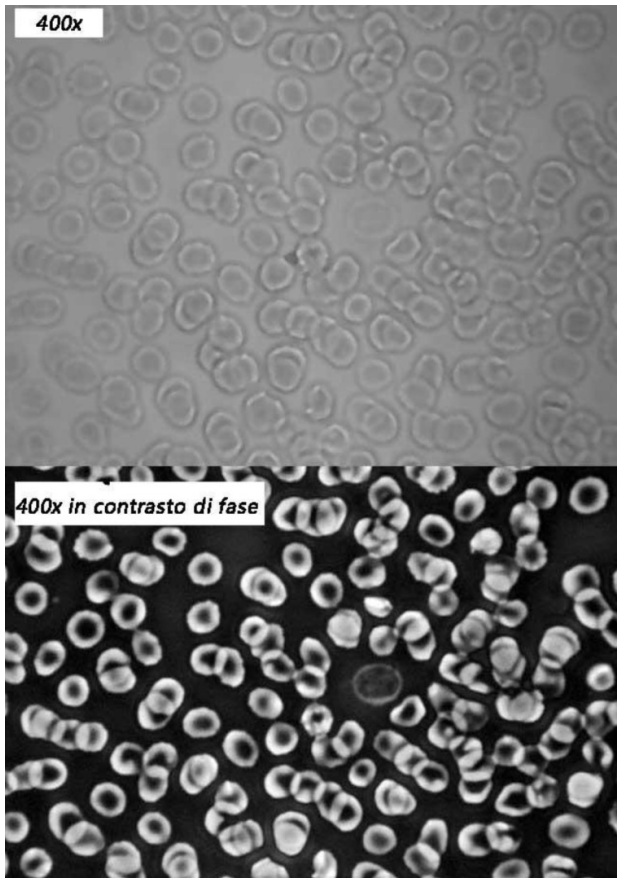


Phase-contrast vs classical microscopy

Phase contrast is especially useful for living biological specimens. Today, cell cultures are a primary specimen for phase contrast. Phase contrast is useful for specimens that produce very little refraction; that is, their refractive index is not much different from their surrounding medium.

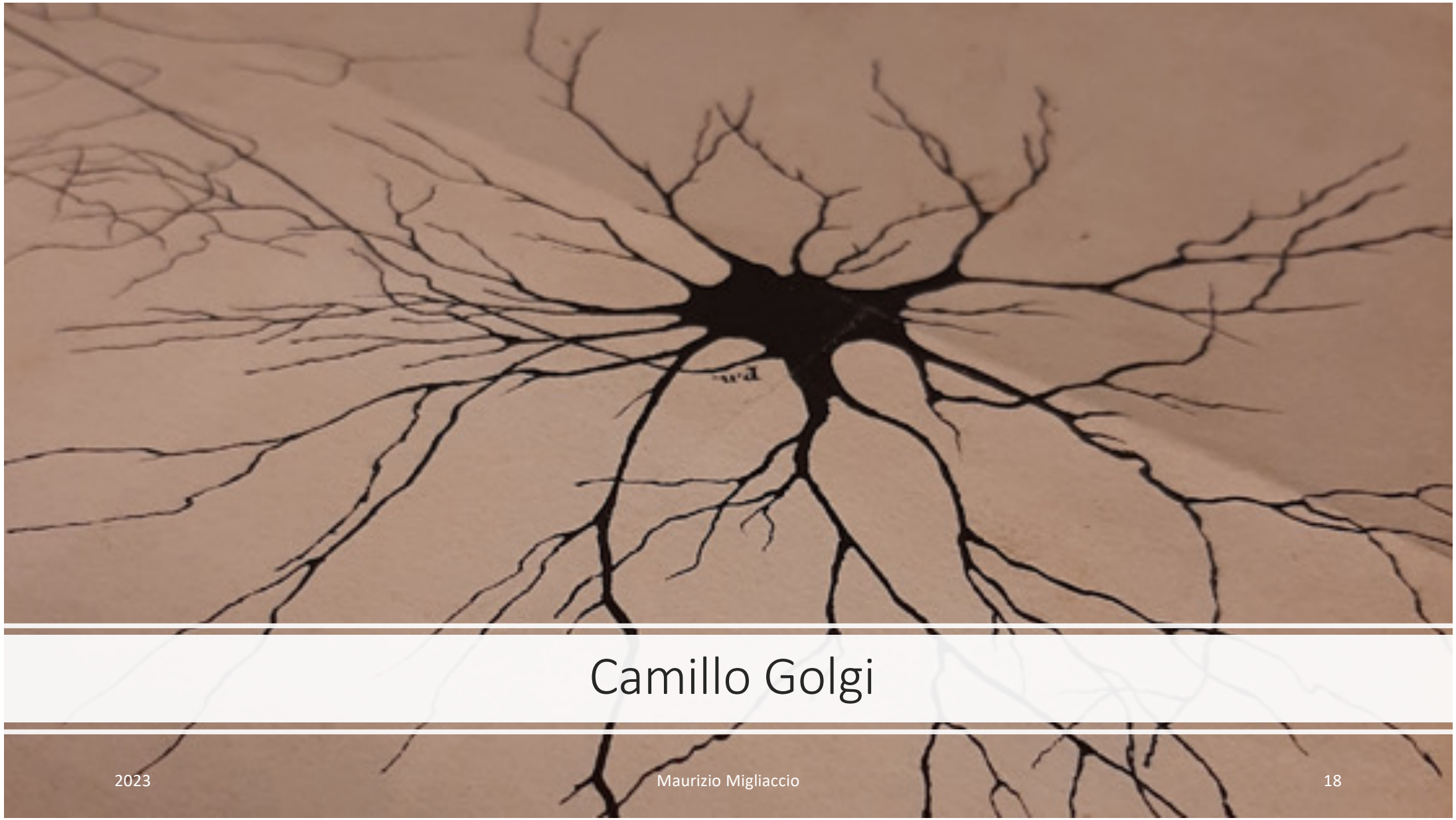
Phase is also useful for specimens that possess little or no color of their own and which have not been artificially colored.

In addition to cell and organ cultures, such specimens include bacteria, aquatic invertebrates, blood, and other body fluids.



Optical microscopes

Two main issues must be considered: spatial resolution and contrast.



Camillo Golgi

Camillo Golgi

- Camillo Golgi (7 July 1843 – 21 January 1926) was an Italian biologist and pathologist known for his works on the central nervous system.
- Golgi and the Spanish biologist Santiago Ramón y Cajal were jointly given the Nobel Prize in Medicine 1906. *First Italian Nobel prize !*
- The Central nervous system was difficult to study during Golgi's time because the cells were hard to identify. The available tissue staining techniques were useless for studying nervous tissue.
- He discovered a method of staining nervous tissue. Since cells are selective stained in black, he called the process la “reazione nera” but today it is called Golgi's method.

Camillo Golgi

- On *16 February 1873*, Camillo Golgi, then director of the *Pio Istituto degli Incurabili of Abbiategrasso*, wrote: "[...] I work many hours under the microscope. I am happy to have found a new reaction to demonstrate even to the blind the structure of the stroma interstitial of the cerebral cortex. I make the silver nitrate act on the pieces of brain hardened in potassium dichromate. I have already obtained very good results and I hope to obtain more [...]".
- This is the first known writing in which Golgi talks about the Black Reaction, a histological technique that allows selective staining of neural cells. The technique therefore allowed him to observe and describe, first of all, the branched shape of the neuron and proved fundamental for the development of neuroscience.


Rai Storia
diXit

Camillo Golgi

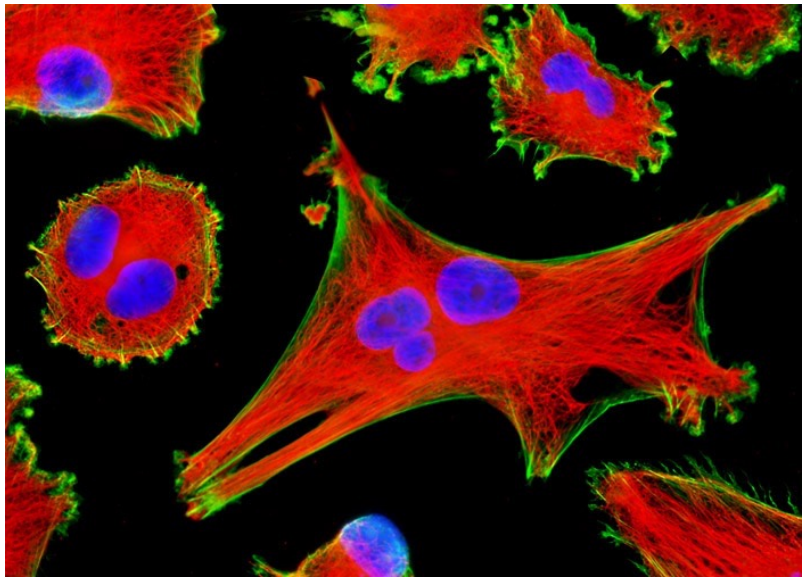
- Camillo Golgi – Nobel diploma. NobelPrize.org. Nobel Prize Outreach AB 2023. Sat. 5 Aug 2023. <https://www.nobelprize.org/prizes/medicine/1906/golgi/diploma/>

Fluorescence microscopy

Fluorescence microscopy

Nikon USED more than 22.000 euro

Human Brain Glioma Cells (U-118 MG Line)



Fluorescence microscopy

- Many of the technical improvements in microscopes over the years have centered on *increasing the contrast* between what is interesting (signal) and what is not (background).
- A fluorescence microscope is an optical microscope used to study organic or inorganic samples by exploiting the phenomena of fluorescence and phosphorescence induced in the sample.
- The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter.

Fluorescence

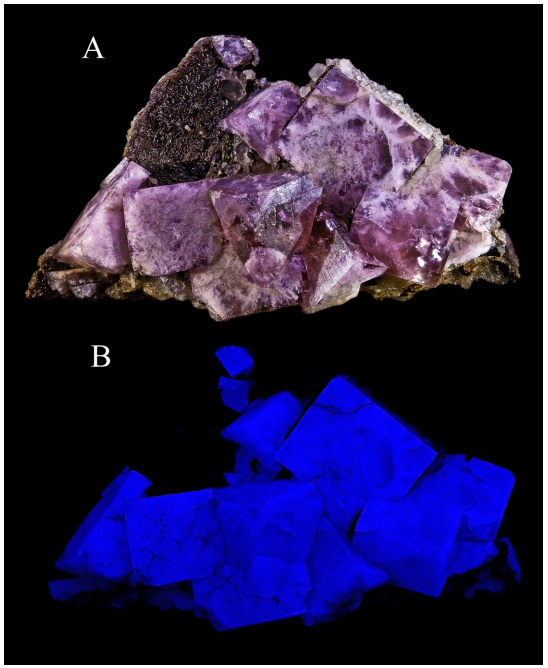
- Fluorescence is the property of some substances to re-emit (in most cases at longer wavelength and therefore lower energy) the electromagnetic radiations received.
- In particular, to absorb radiations in the ultraviolet and emit them in the visible.

Fluorescent clothes used in black light theater production, Prague

Fluorescence

- George Gabriel Stokes named the phenomenon of fluorescence from fluorite, in 1852.
- Many samples of fluorite exhibit fluorescence under ultraviolet light, a property that takes its name from fluorite.
- Fluorescence involves the elevation of electron energy levels by quanta of ultraviolet light, followed by the progressive falling back of the electrons into their previous energy state, releasing quanta of visible light in the process. In fluorite, the visible light emitted is most commonly blue, but red, purple, yellow, green, and white also occur. The fluorescence of fluorite may be due to mineral impurities, such as yttrium and ytterbium, or organic matter, such as volatile hydrocarbons in the crystal lattice.

Fluorescing fluorite from Boltsburn Mine, Weardale, North Pennines, County Durham, England, UK.



George Gabriel Stokes

- Sir George Gabriel Stokes, 1st Baronet, (13 August 1819 – 1 February 1903) was an Irish physicist and mathematician.
- As a physicist, Stokes made seminal contributions to fluid mechanics, including the Navier–Stokes equations; and to physical optics, with notable works on polarization and fluorescence.

Fluorescence

- Gregorio Weber (4 July 1916 – 18 July 1997) was an Argentinian scientist who made significant contributions to the fields of fluorescence spectroscopy and protein chemistry.
- His thesis, titled "Fluorescence of Riboflavin, Diaphorase and Related Substances", marked the beginning of the application of fluorescence spectroscopy to biomolecules.
- Osamu Shimomura (August 27, 1928 – October 19, 2018) was a Japanese organic chemist and marine biologist, and professor emeritus at Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts and Boston University School of Medicine.
- He was awarded the Nobel Prize in Chemistry in 2008 for the discovery and development of green fluorescent protein (GFP) with two American scientists: Martin Chalfie of Columbia University and Roger Tsien of the University of California-San Diego.

Fluorescence

- Fluorescent protein: Processes such as the development of nerve cells in the brain or the growth of cancer cells would have remained hidden and invisible without the fluorescent protein. The protein is called GFP (Green Fluorescent Protein) and was observed for the first time in 1962 in a jellyfish, Aequorea victoria.

MBL

The Marine Biological Laboratory (MBL) is an international center for research and education in biological and environmental science.

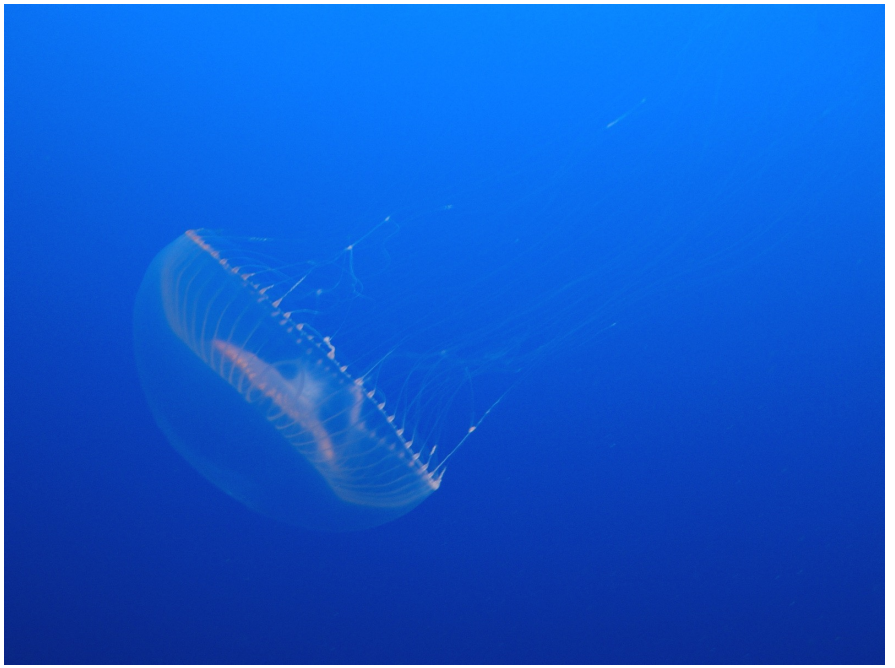
Founded in Woods Hole, Massachusetts, in 1888, the MBL is a private, nonprofit institution that was independent for most of its history, but became officially affiliated with the University of Chicago on July 1, 2013.

As of 2023, 60 Nobel Prize winners have been affiliated with MBL as students, faculty members or researchers

Fluorescence

- Due to the potential for widespread usage and the evolving needs of researchers, *many different mutants of GFP have been engineered*.
- The first major improvement was a single point mutation (S65T) reported in 1995 in Nature by Roger Tsien. This mutation dramatically improved the spectral characteristics of GFP, resulting in increased fluorescence, photostability, and a shift of the major excitation peak to 488 nm, with the peak emission kept at 509 nm.
- Many other mutations have been made, including color mutants.

Aequorea victoria



Fluorescence microscopy

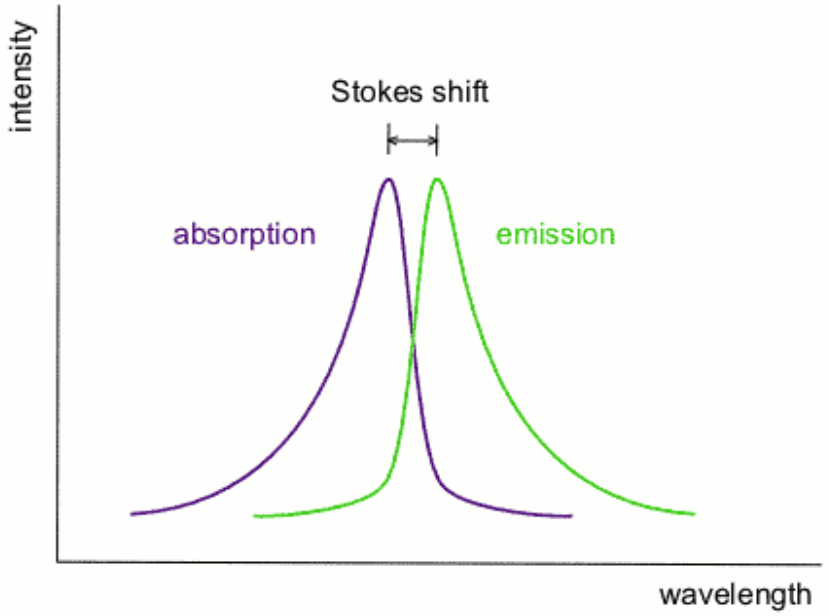
- Fluorescence microscopy is the quintessential example, as it aims to reveal only the objects of interest in an otherwise black background.
- Because of its intrinsic selectivity, fluorescence imaging has become the mainstay of microscopy in the service of biology.
- Over the past several decades, organic chemists have devised many thousands of fluorescent probes that provide a means of labeling virtually any imaginable aspect of biological systems.

Fluorescence microscopy

- For example, the Molecular Probes Handbook, tenth edition online (http://probes.invitrogen.com/handbook/), which is perhaps the largest compendia of fluorescence applications for biologists, has 23 chapters, 14 of which describe the 3,000 or so fluorescent probes for a wide range of cell biological questions.
- The large spectral range of available fluorophores allows simultaneous imaging of different cellular, subcellular or molecular components.

Fluorescence microscopy

- Fluorescence microscopy requires that the objects of interest fluoresce.
- Fluorescence is the emission of light that occurs within nanoseconds after the absorption of light that is typically of shorter wavelength.
- The difference between the exciting and emitted wavelengths, known as the Stokes shift, is the critical property that makes fluorescence so powerful.
- By completely filtering out the exciting light without blocking the emitted fluorescence, it is possible to see only the objects that are fluorescent.



Fluorescence microscopy

- This approach to contrast is superior to absorption techniques in which objects are stained with agents that absorb light.
- With absorbance dyes, the amount of light absorbed becomes only infinitesimally different from the background for small objects.
- In fluorescence, however, even single fluorescent molecules are visible if the background has no autofluorescence.

Fluorescence microscopy

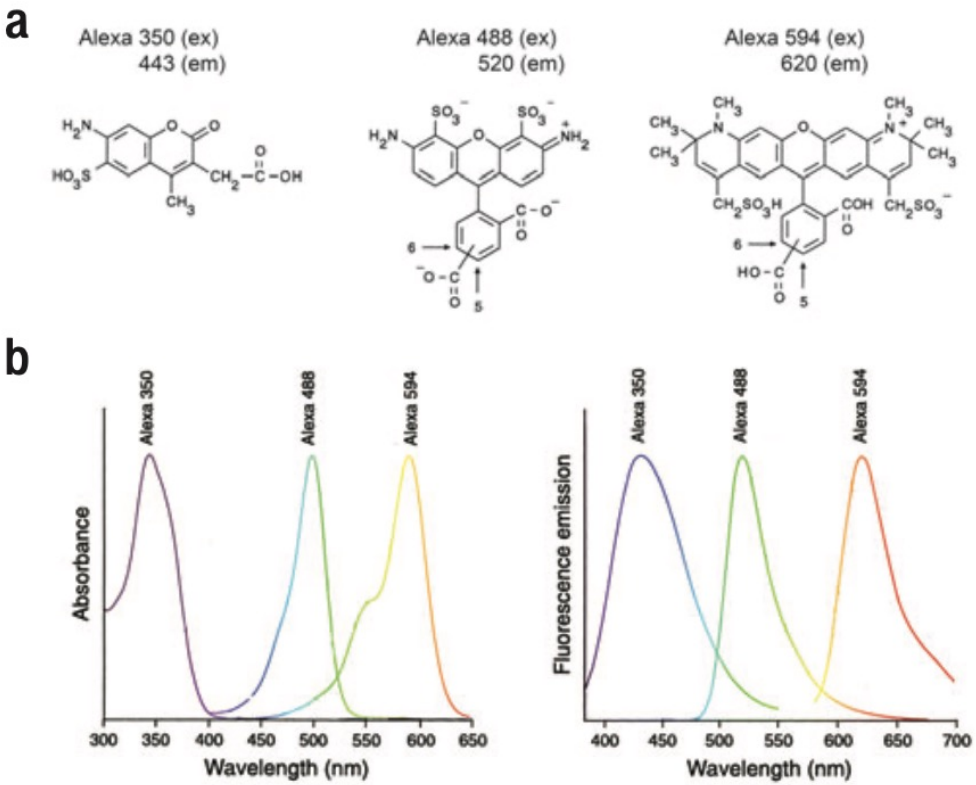
- Molecules that are used by virtue of their fluorescent properties are called fluorophores.
- The outermost electron orbitals in the fluorophore molecule determine both its efficiency as a fluorescent compound and the wavelengths of absorption and emission.
- When fluorescent compounds in their so-called ‘ground state’ absorb light energy (photons), alterations in the electronic, vibrational and rotational states of the molecule can occur.
- The absorbed energy sometimes moves an electron into a different orbital that is on average farther away from the nucleus. This transition to an ‘excited state’ occurs very rapidly (in femtoseconds).

Fluorescence microscopy

A more serious overlap concern relates to imaging multiple fluorophores in the same sample.

Given the broad excitation and emission spectra of each fluorophore, even spectrally shifted fluorophores can be excited by the same wavelength and exhibit overlapping emission.

These overlaps can cause confusing crosstalk or bleed-through between signals associated with different fluorophores in the same sample. This difficulty has been ameliorated by organic synthesis of ever wider spectral ranges of fluorophores (by adding more conjugated bonds) that now permit a user to choose fluorophores with little overlap in excitation and/or emission spectra (**Fig. a**,**b**).

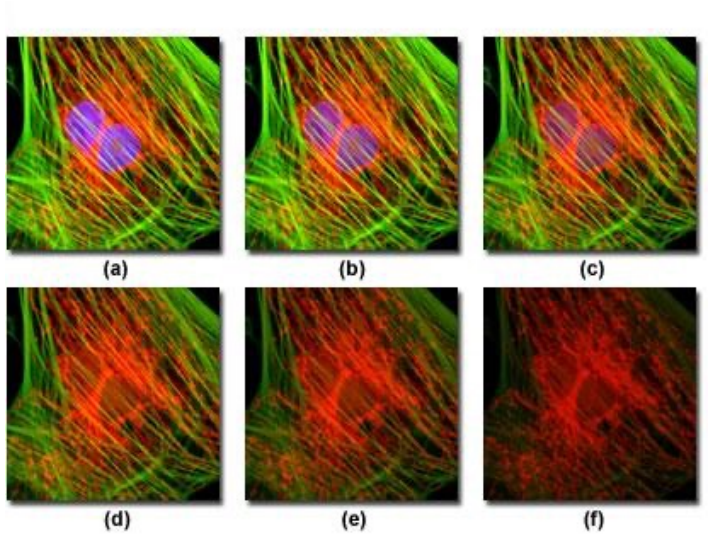


Fluorescence microscopy

The phenomenon of bleaching

- Although in principle a fluorophore can cycle between ground and excited states an unlimited number of times, the conditions in which organic fluorophores are used usually limit the number of cycles.
- Estimates of 10,000–40,000 cycles are often cited as the limit before permanent bleaching occurs for good fluorophores.
- Bleaching is a generic term for all of the processes that cause the fluorescent signal to fade permanently.

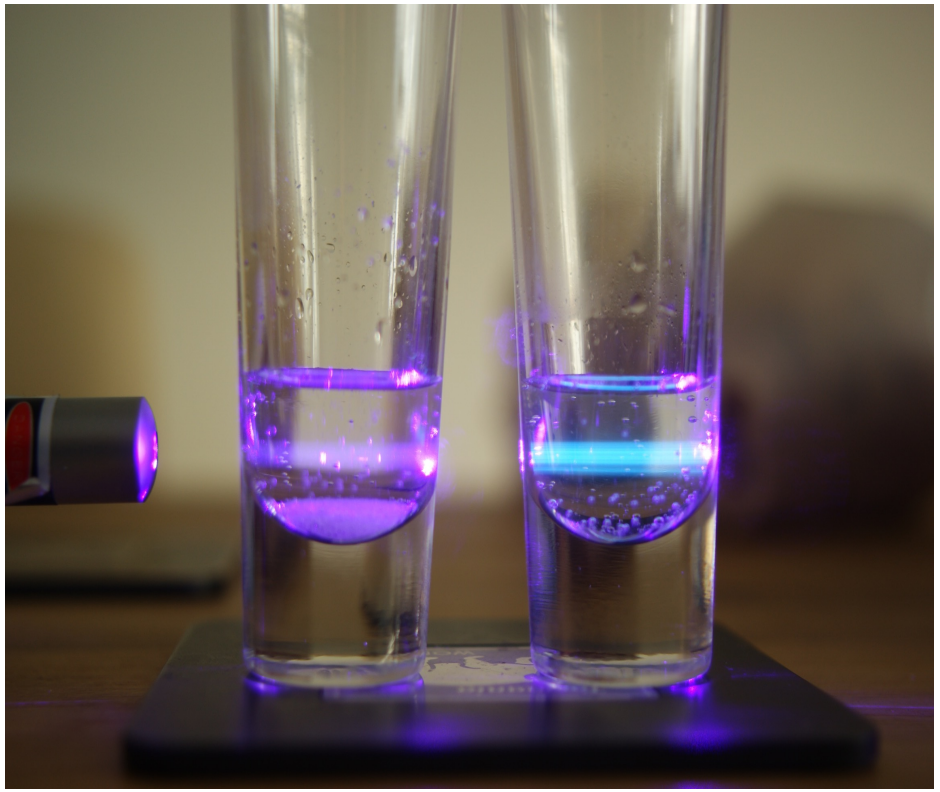
The figure shows a typical example of bleaching observed in a series of digital images captured at different times relative to a culture of cells.



Fluorescence microscopy

The phenomenon of quenching

- Quenching on the other hand, is a reversible loss of fluorescence owing to noncovalent interactions between a fluorophore and its molecular milieu.
- Quenching is often heavily dependent on pressure and temperature.

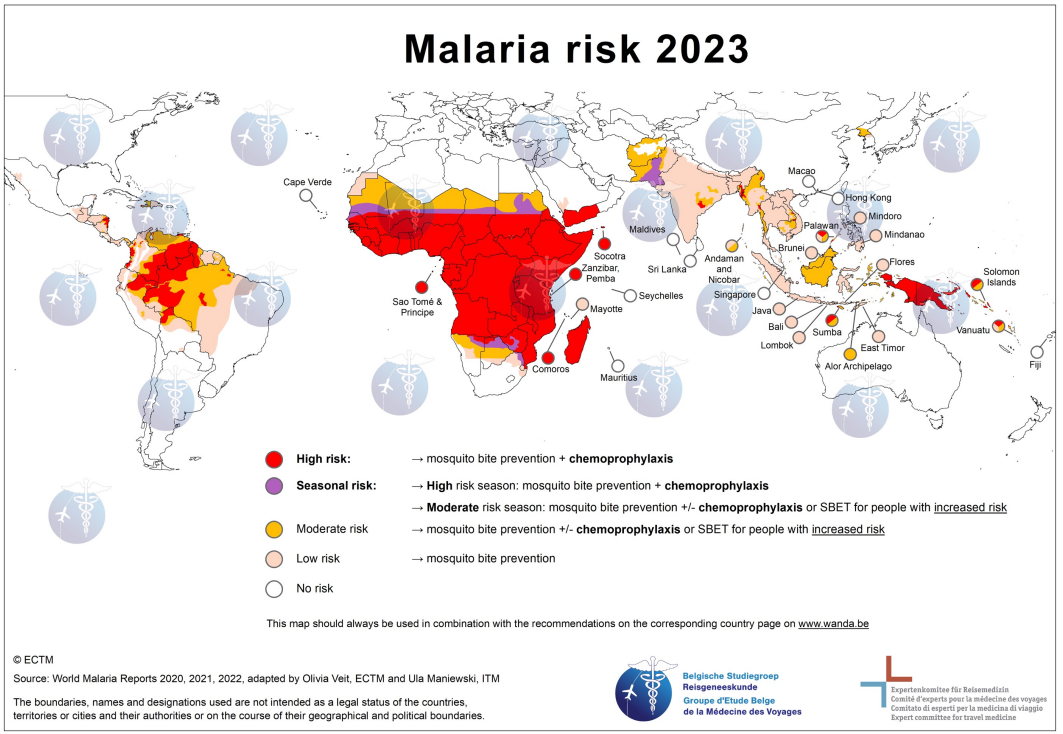


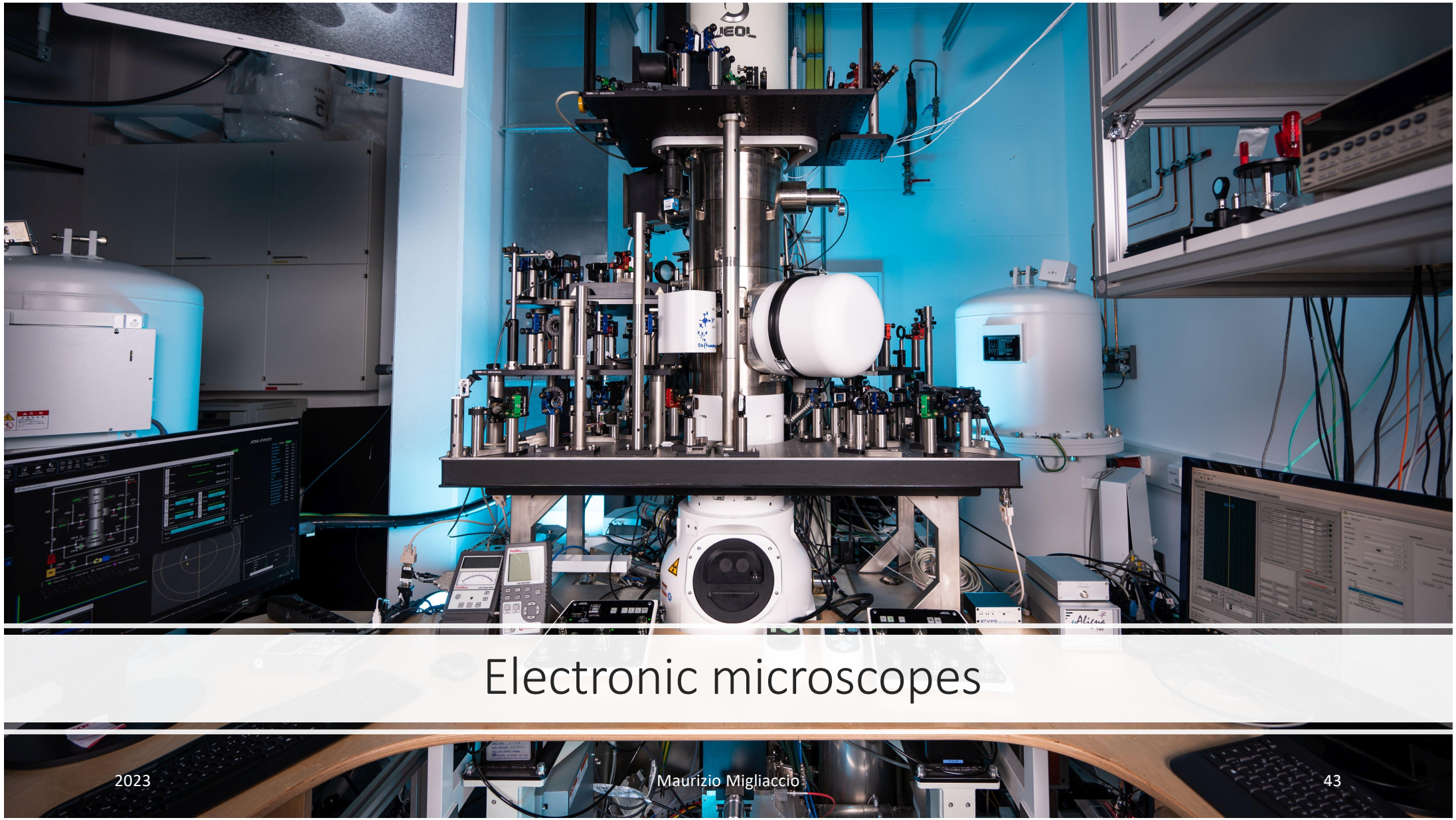
WHO

- Fluorescence microscopy has proved to be a useful and cost-effective procedure for surveillance of disease outbreaks and for diagnosis of a wide range of both communicable and noncommunicable diseases.
- More recently, less expensive fluorescence microscopes have been developed, and accessories now are available that convert an existing bright-field microscope into a fluorescence microscope.

WHO

Fluorescent staining is commonly used to improve tuberculosis diagnosis efficiency as well as for malaria diagnosis, for early detection of bacteria in blood cultures, and to detect and identify nucleic acids by colour.

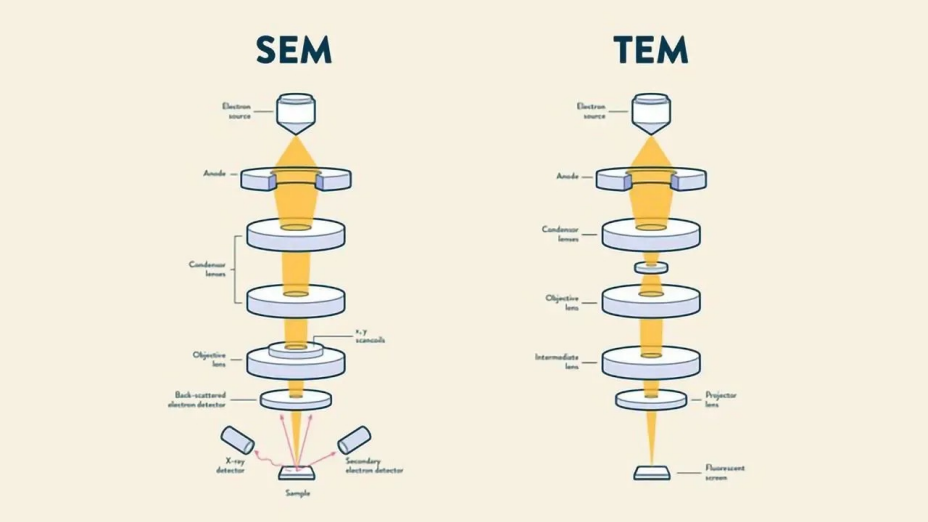




Electronic microscopes

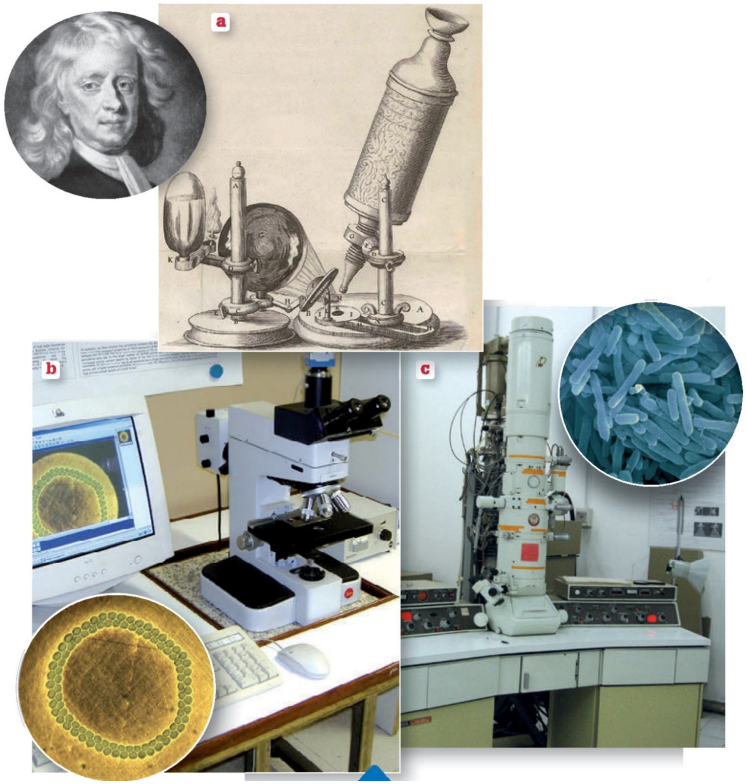
Electron microscopes

- Electron microscopes have emerged as a powerful tool for the characterization of a wide range of materials.
- Their versatility and extremely high spatial resolution render them a very valuable tool for many applications.
- The two main types of electron microscopes are the transmission electron microscope (TEM) and the scanning electron microscope (SEM).



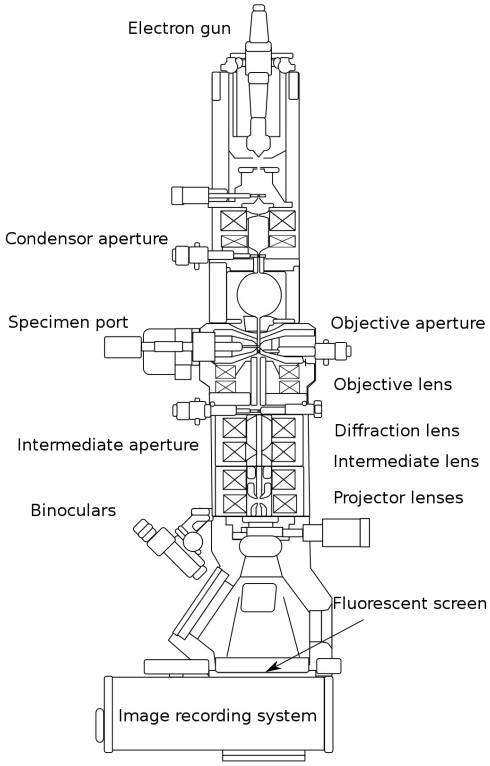
Electron microscopes

- (a) Historical representation of the optical microscope by R. Hooke (London, 1664).
- The optical microscope (b) is essential for a quick analysis of the samples. Distances of the order of µm can be easily appreciated on planar samples. The maximum magnification available is approx 4.000x.
- The TEM (c) operates up to 200 kV and can reach a resolution of 2 Angstrom by using ultrathin samples.
- In the photo, station with an electron microscope and scanning of Natrialba magadii, an extremophile bacterium, which together with Deinococcus radiodurans has proved to be the only one to resist cosmic radiation.



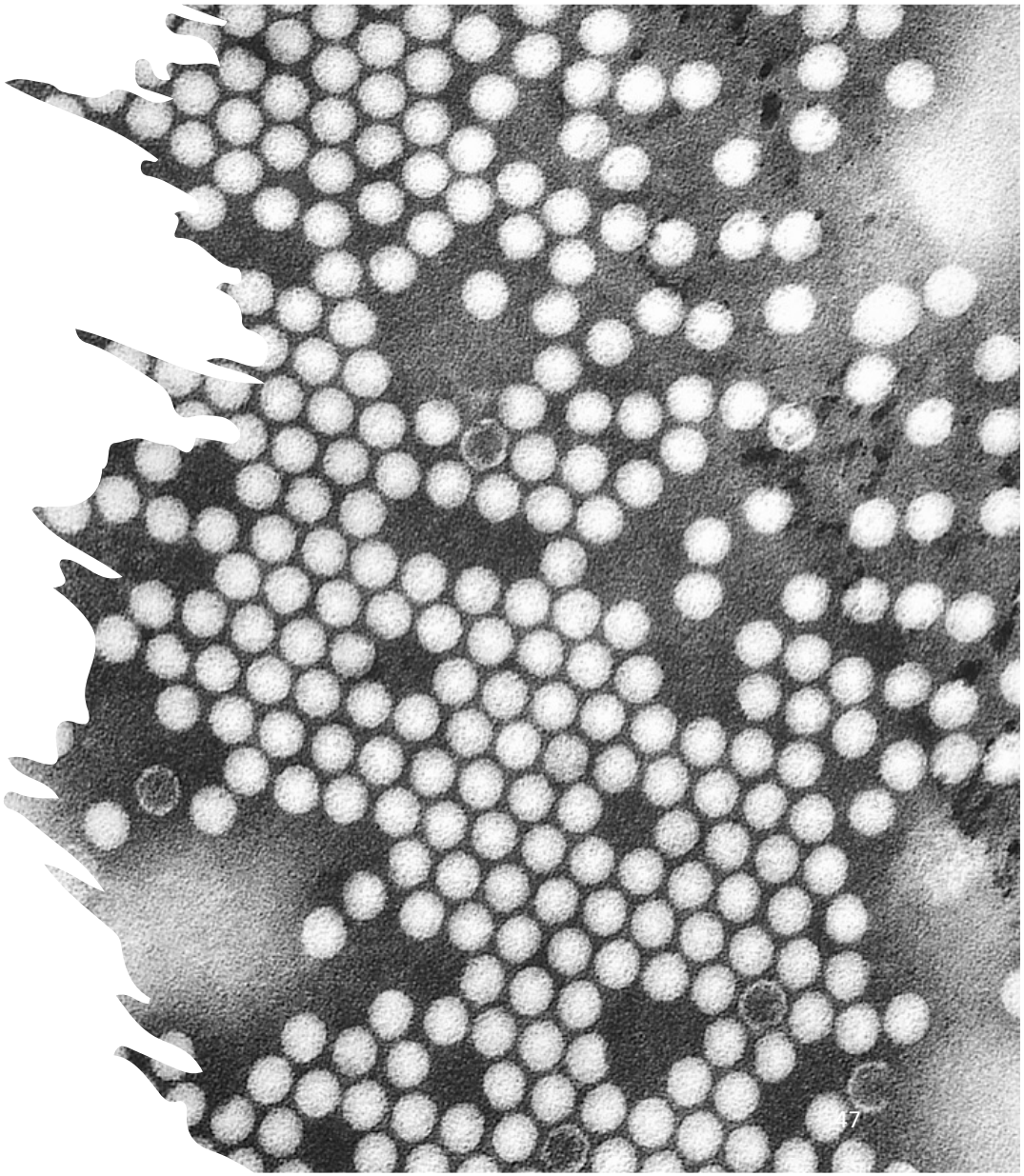
TEM

- Transmission electron microscopy (TEM) is a microscopy technique in which a beam of electrons is transmitted through a specimen to form an image.
- The specimen is usually an ultrathin section less than 100 nm thick or a suspension on a grid.
- An image is formed from the interaction of the electrons with the sample as the beam is transmitted through the specimen.
- The image is then magnified (TEMs can magnify objects up to 2 million times) and focused onto an imaging device.



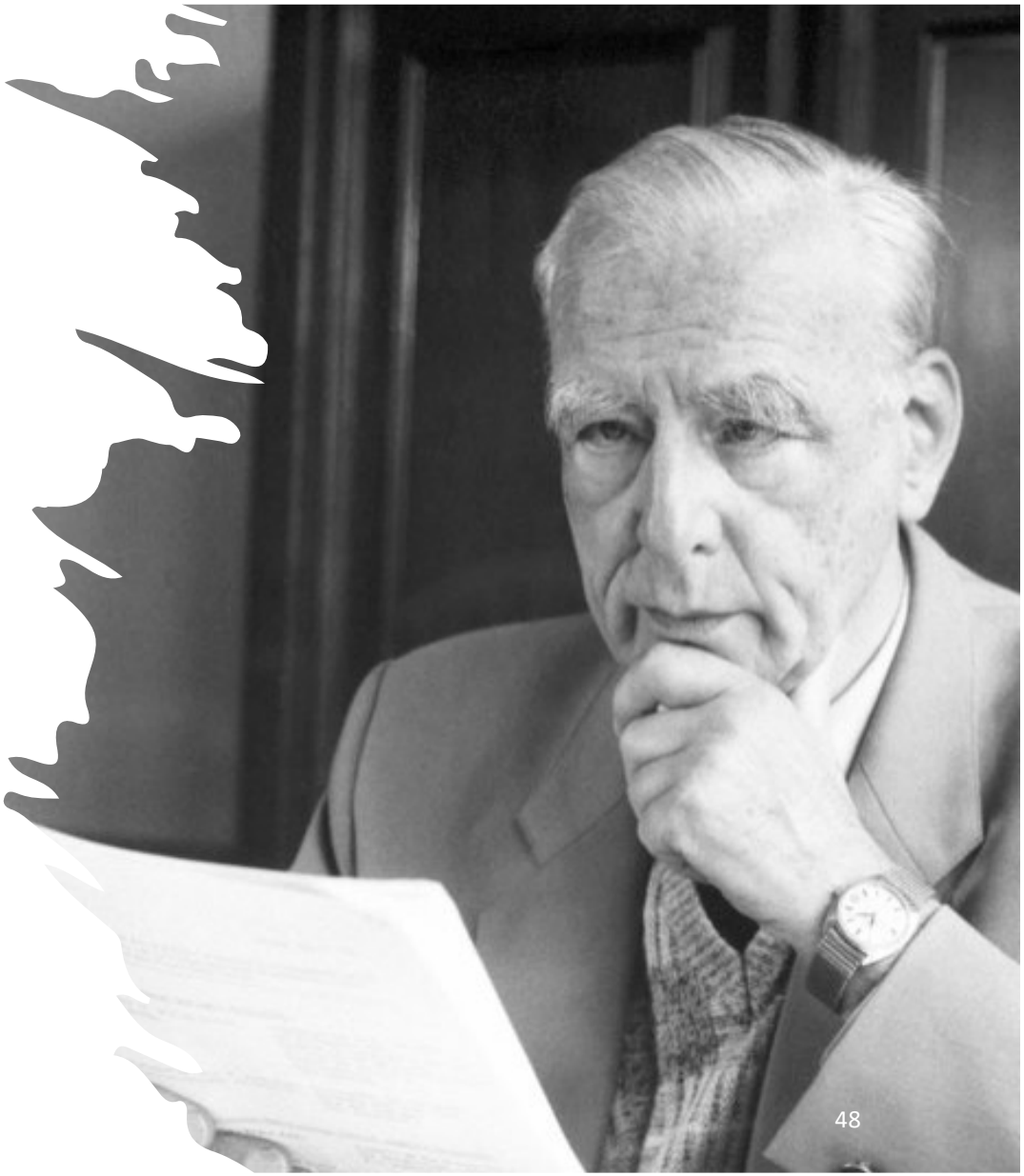
TEM

- Transmission electron microscopes are capable of imaging at a significantly higher resolution than light microscopes, owing to the smaller de Broglie wavelength of electrons.
- The first TEM was demonstrated by Max Knoll and Ernst Ruska in 1931, with this group developing the first TEM with resolution greater than that of light in 1933 and the first commercial TEM in 1939. *In 1986, Ruska was awarded the Nobel Prize in physics for the development of transmission electron microscopy*.



Ernst Ruska

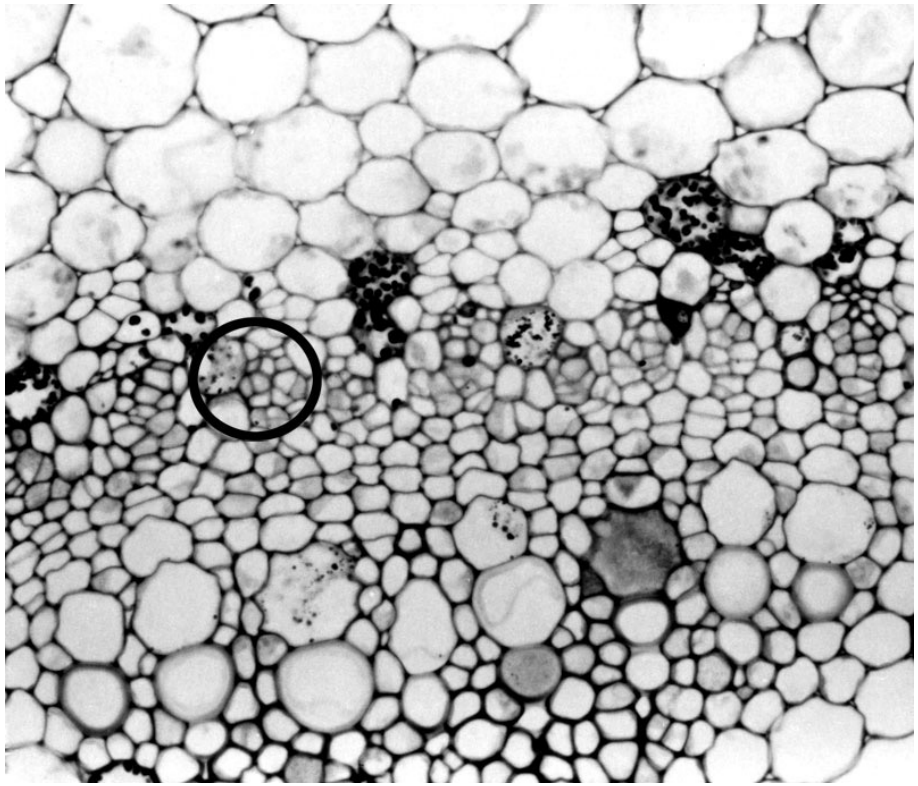
- Ernst August Friedrich Ruska (25 December 1906 – 27 May 1988) was a German physicist who won the Nobel Prize in Physics in 1986 for his work in electron optics, including the design of the first electron microscope.

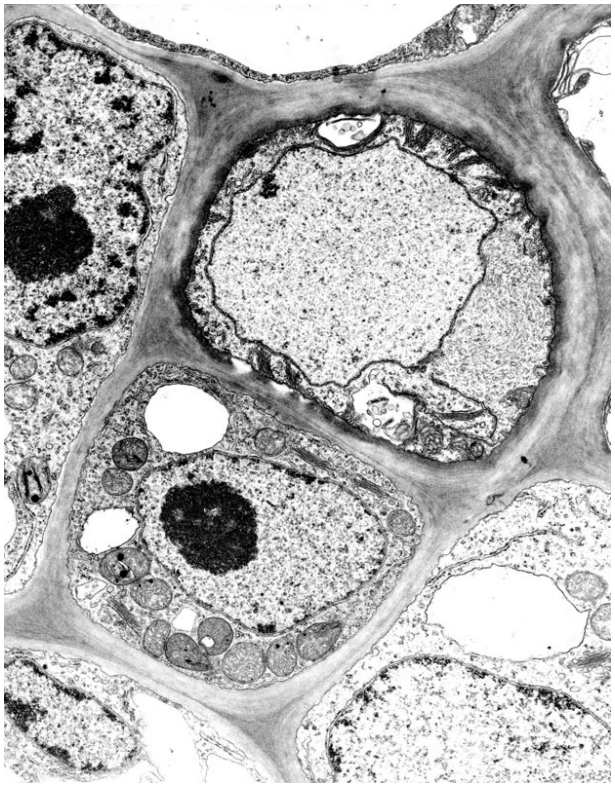


TEM vs optical

- Although TEMs and light microscopes operate on the same basic principles, there are several differences between the two.
- The main difference is that TEMs use electrons rather than light in order to magnify images.
- The power of the light microscope is limited by the wavelength of light and can magnify something up to 2,000 times.
- Electron microscopes, on the other hand, can produce much more highly magnified images because the beam of electrons has a smaller wavelength which creates images of higher resolution. (Resolution is the degree of sharpness of an image.)
- In next image a comparison of the magnification of a light microscope to that of a TEM is shown.

TEM vs optical





TEM

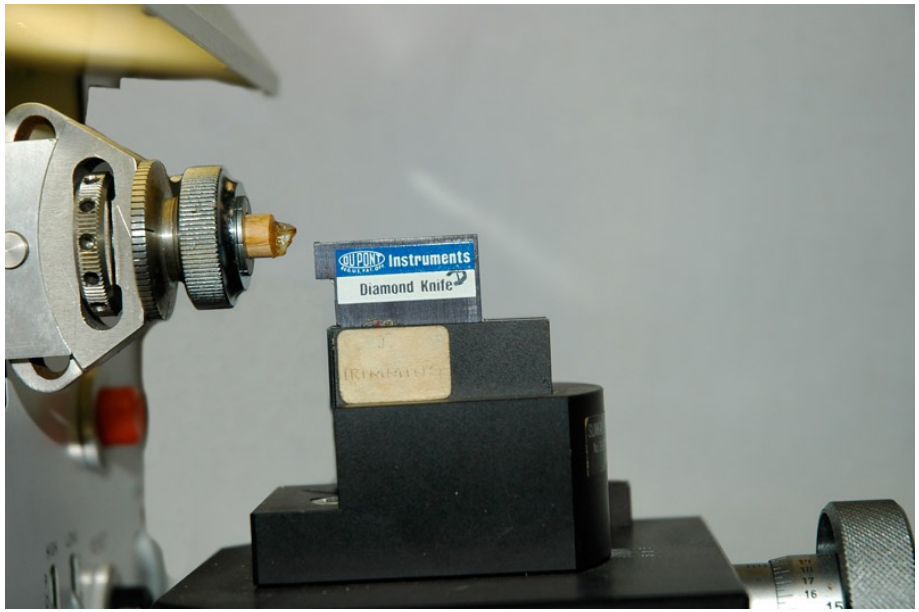
How are TEM specimens prepared?

Specimens must be very thin so that electrons are able to pass through the tissue.

This may be done by cutting very thin slices of a specimen's tissue using an ultramicrotome.

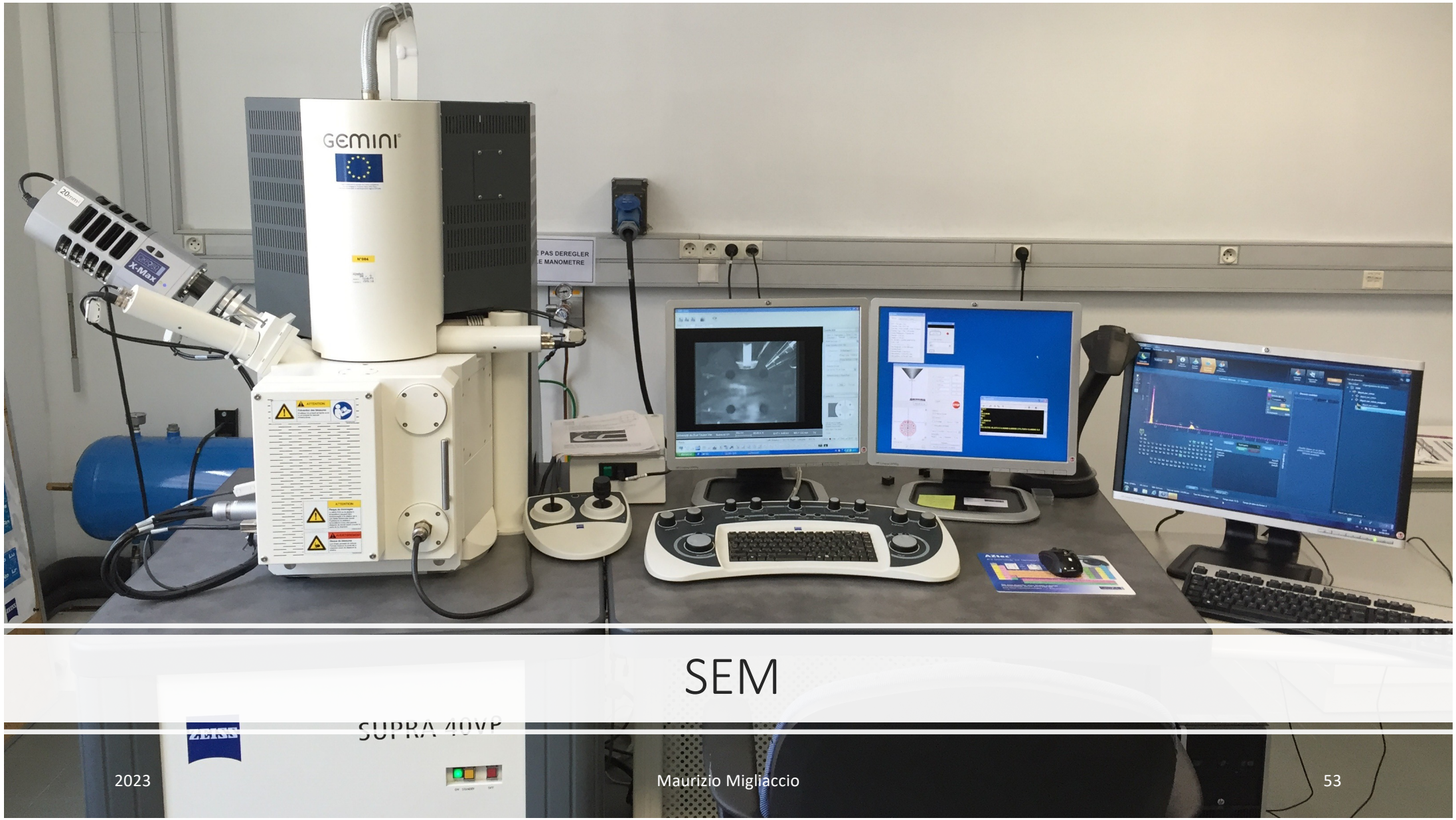
The tissue must first be put in a chemical solution to preserve the cell structure.

The tissue must also be completely dehydrated (all water removed).



TEM

- The price of an Electron Microscope varies dramatically.
- Transmission Electron Microscopes (TEM) cost $100,000 to $10,000,000 for new and $125,000 to $900,000 for used instruments.
- The Hitachi HT7800 RuliTEM is a 120 kV transmission electron microscope (TEM). ☞
- Its intermediate 7820 version has a 0.14 nm resolution and a maximum magnification equal to x800,000



SEM

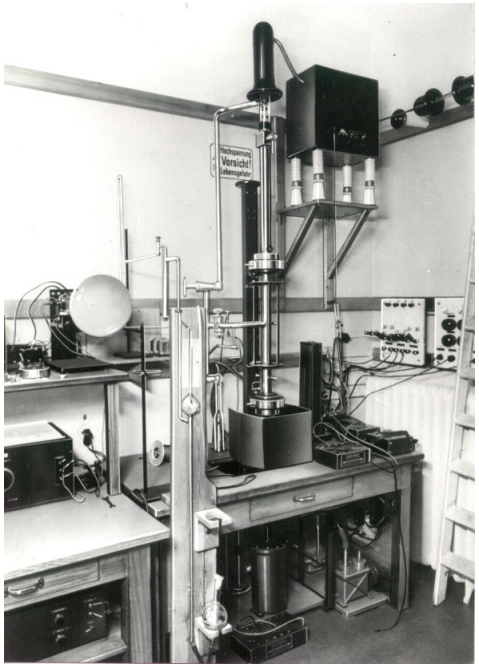
Manfred von Ardenne

- Manfred baron von Ardenne (20 January 1907 – 26 May 1997) was a German researcher and applied physicist and inventor.
- He took out approximately 600 patents in fields including electron microscopy, medical technology, etc.

SEM

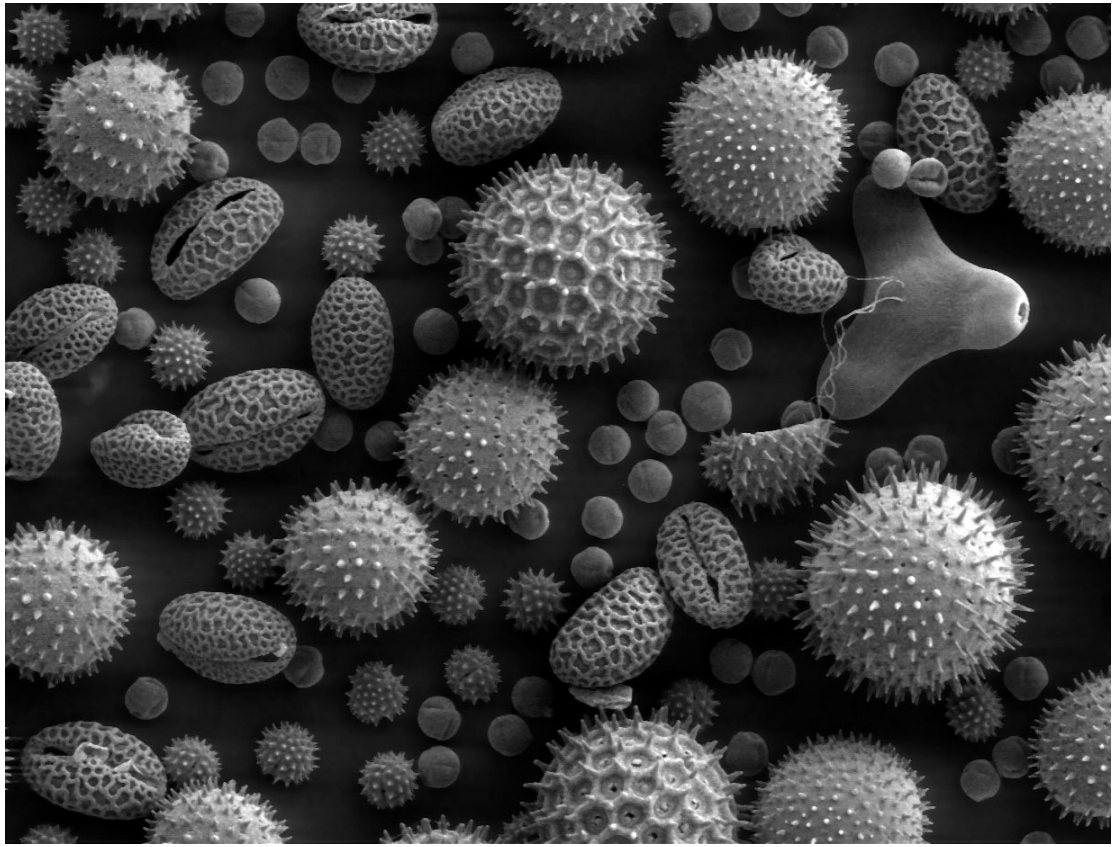
- A Scanning Electron Microscope (SEM) is a type of electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons.
- The electrons interact with atoms in the sample, producing various signals that contain information about the surface topography and composition of the sample.

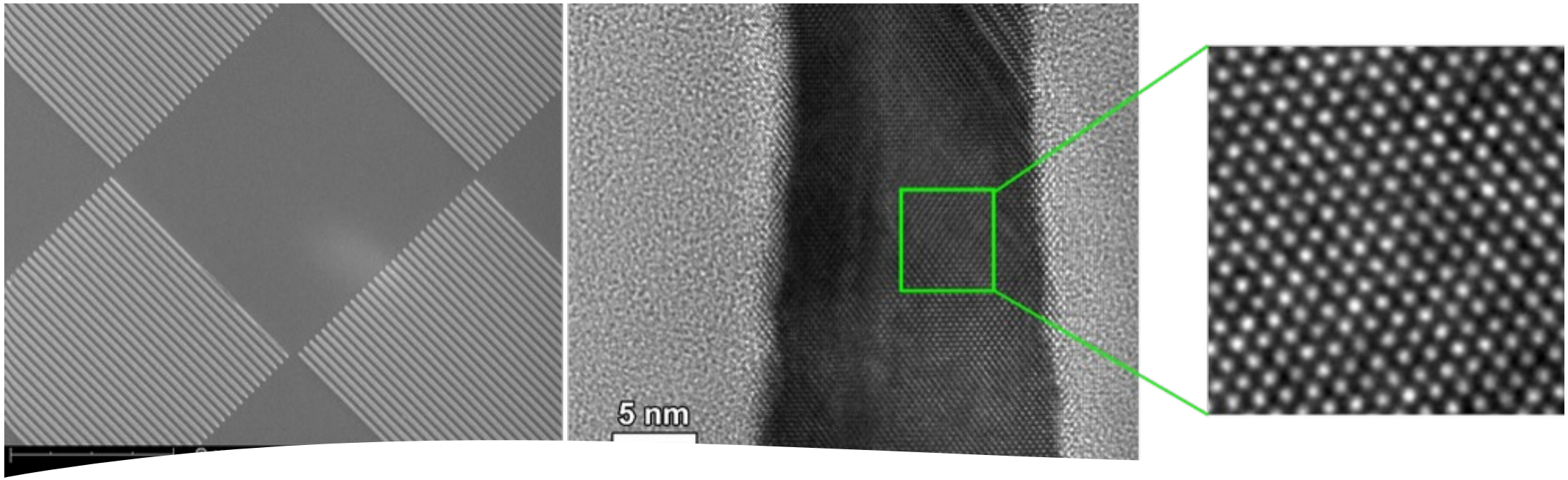
M. von Ardenne's first SEM



SEM

Image of pollen grains taken on a SEM shows the characteristic depth of field of SEM micrographs





TEM vs SEM

- The main difference between SEM and TEM is that SEM creates an image by detecting reflected or knocked-off electrons, while TEM uses transmitted electrons (electrons that are passing through the sample) to create an image.
- As a result, TEM offers valuable information on the inner structure of the sample, such as crystal structure, morphology and stress state information, while SEM provides information on the sample's surface and its composition.
- Electron microscopy images of silicon. a) SEM image with SED offers information on the morphology of the surface, while b) TEM image reveals structural information about the inner sample.

TEM vs SEM

For both techniques, electrons are used to acquire images of samples. Their main components are the same:

- An electron source
- A series of electromagnetic and electrostatic lenses to control the shape and trajectory of the electron beam
- Electron apertures

All of these components are housed inside a chamber that is under high vacuum.

TEM vs SEM

- Now for the differences. SEMs use a specific set of coils to scan the beam in a raster-like pattern and collect the scattered electrons.
- The transmission electron microscopy (TEM) principle, as the name suggests, is to use the *transmitted electrons*, the electrons that are passing through the sample before they are collected. As a result, TEM offers invaluable information on the inner structure of the sample, such as crystal structure, morphology, and stress state information, while SEM provides information on the sample's surface and its composition.
- Moreover, one of the most pronounced differences between the two methods is the optimal spatial resolution that they can achieve. *SEM resolution is limited to ~0.5 nm (1nm = 10^{-9} m), while with the recent development in aberration-corrected TEMs, images with spatial resolution of even less than 50 pm (1pm = 10^{-12} m) have been reported.*

TEM vs SEM

Which electron microscopy technique is best for your analysis?

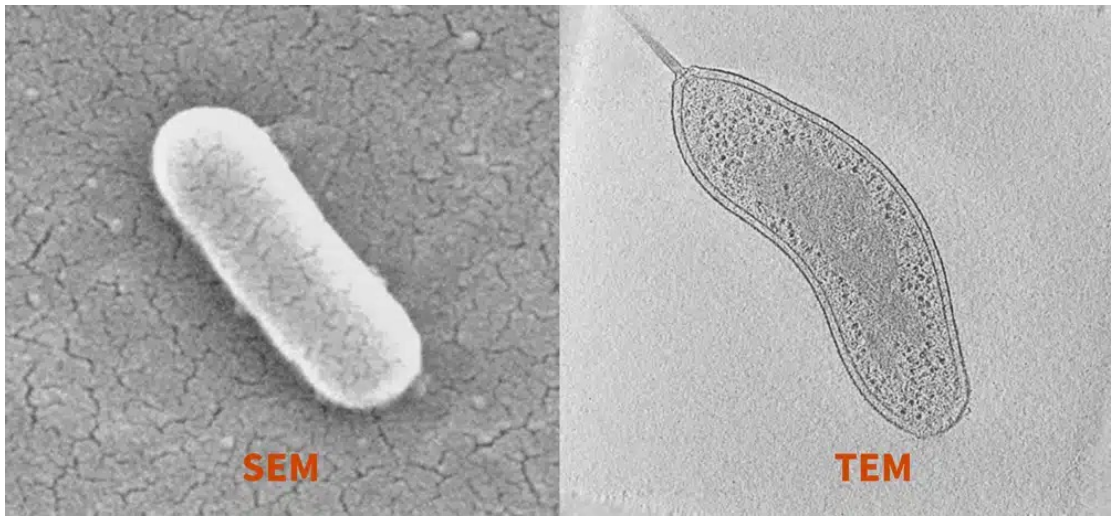
- This all depends on what type of analysis you want to perform. For example, if you want to get information on the surface of your sample, like roughness or contamination detection, then you should choose a SEM.
- On the other hand, if you would like to know what the crystal structure of your sample is, or if you want to look for possible structural defects or impurities, then using a TEM is the only way to do so.

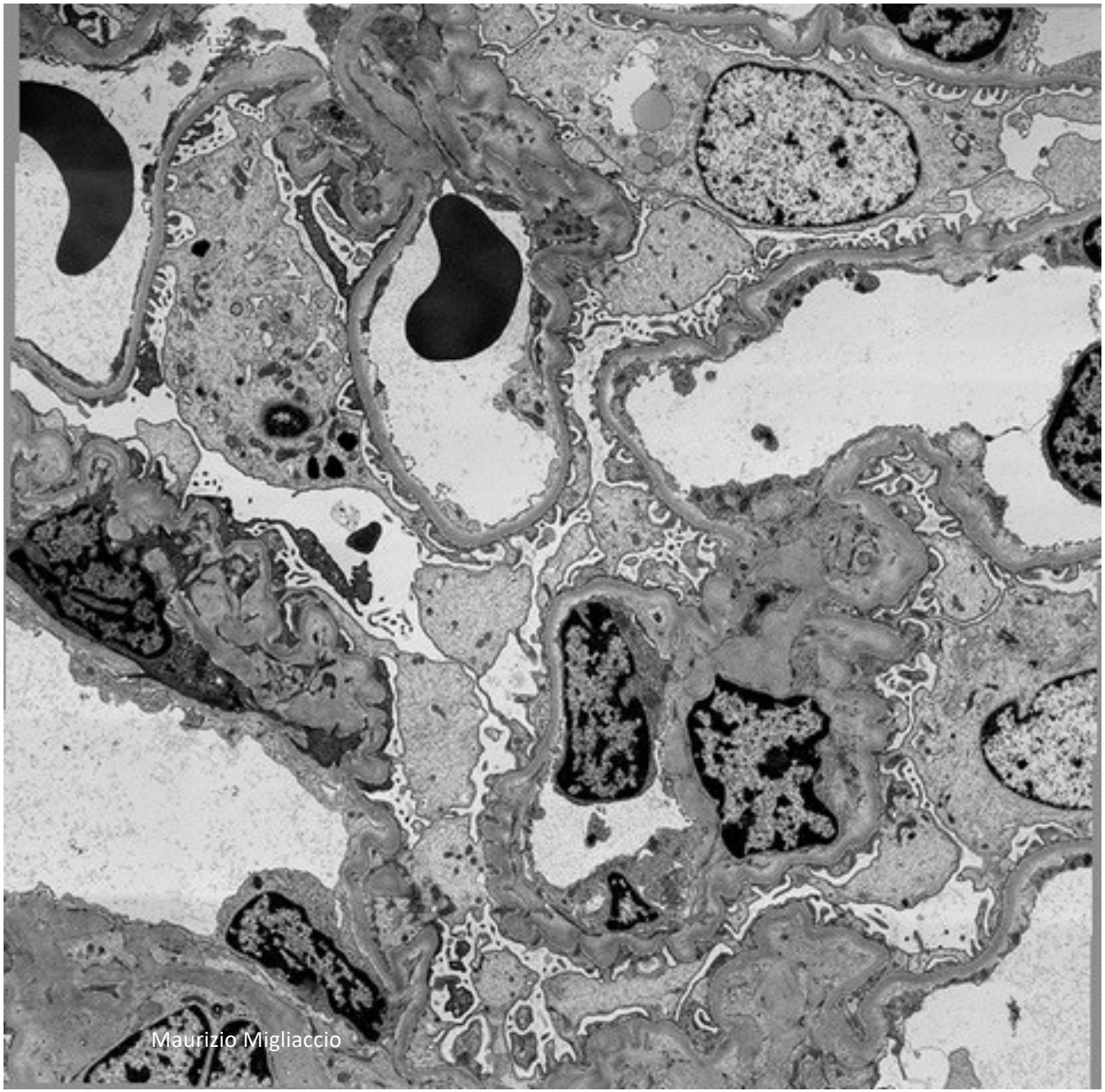
TEM vs SEM

- Due to the requirement for transmitted electrons, TEM samples must be very thin (generally less than 150 nm) and in cases that high-resolution imaging is required, even below 30 nm, whereas for SEM imaging, there is no such specific requirement.
- This reveals one more major difference between the two techniques: sample preparation. SEM samples require little or no effort for sample preparation and can be directly imaged by mounting them on an aluminum stub.
- In contrast, TEM sample preparation is a quite complex and tedious procedure that only trained and experienced users can follow successfully. The samples need to be very thin, as flat as possible, and the preparation technique should not introduce any artifacts (such as precipitates or amorphization) to the sample. Many methods have been developed, including electropolishing, mechanical polishing, and focused ion beam milling. Dedicated grids and holders are used to mount the TEM samples.

TEM vs SEM

SEM provides a 3D image of the surface of the sample, whereas TEM images are 2D projections of the sample, which in some cases makes the interpretation of the results more difficult for the operator.



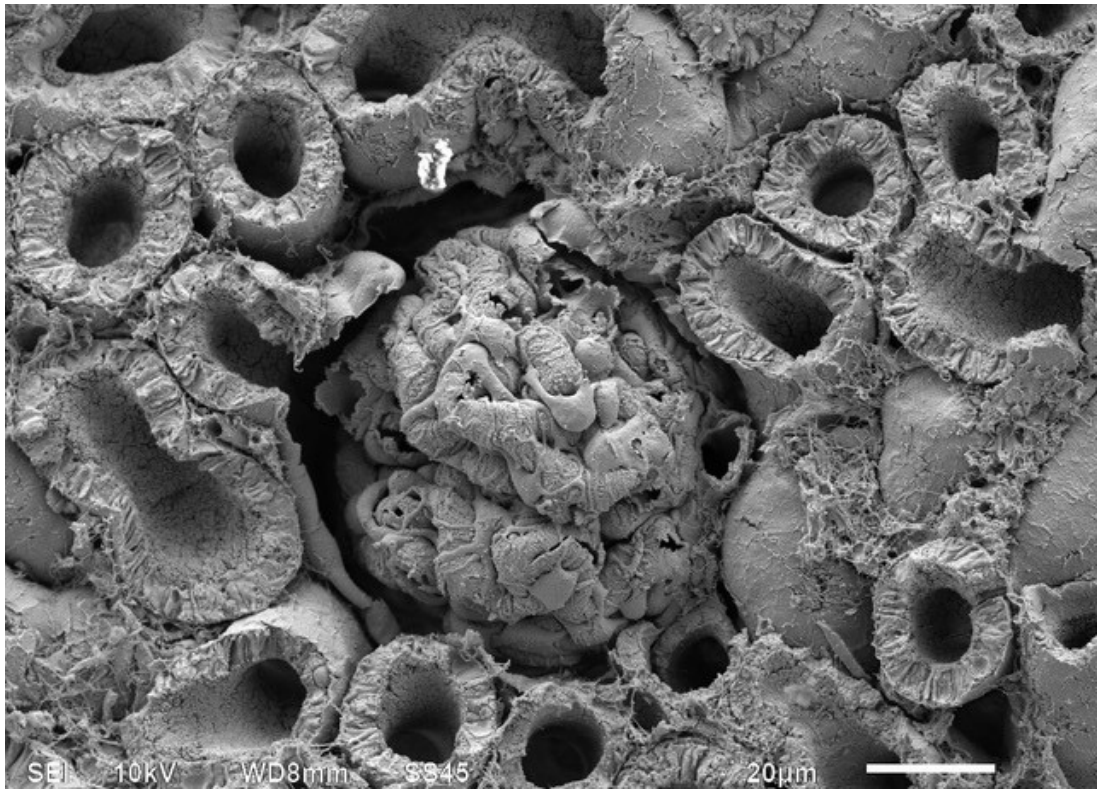


TEM

- TEM image of kidney tissue

SEM

SEM image of kidney tissue.



An undescribed species of Reichenbachia (short-winged mold beetle, Staphylinidae: Pselaphinae), SEM image by Michael Caterino @SBMNH.

SEM

For an advanced SEM, depending on your needs and the technology specifications, you can expect to pay $150,000 to well over $1,000,000.

Purchasing a high-quality refurbished SEM is a much less expensive option: ranges in price from $65,000 – $150,000.

- ZEISS SEM Gemini 500 ☞

Resolution: 0.5 nm at 15 kV; 0.9 nm at 1 kV, 1.0 nm at 500 V.

Magnification: 50 times to 2,000,000 times.

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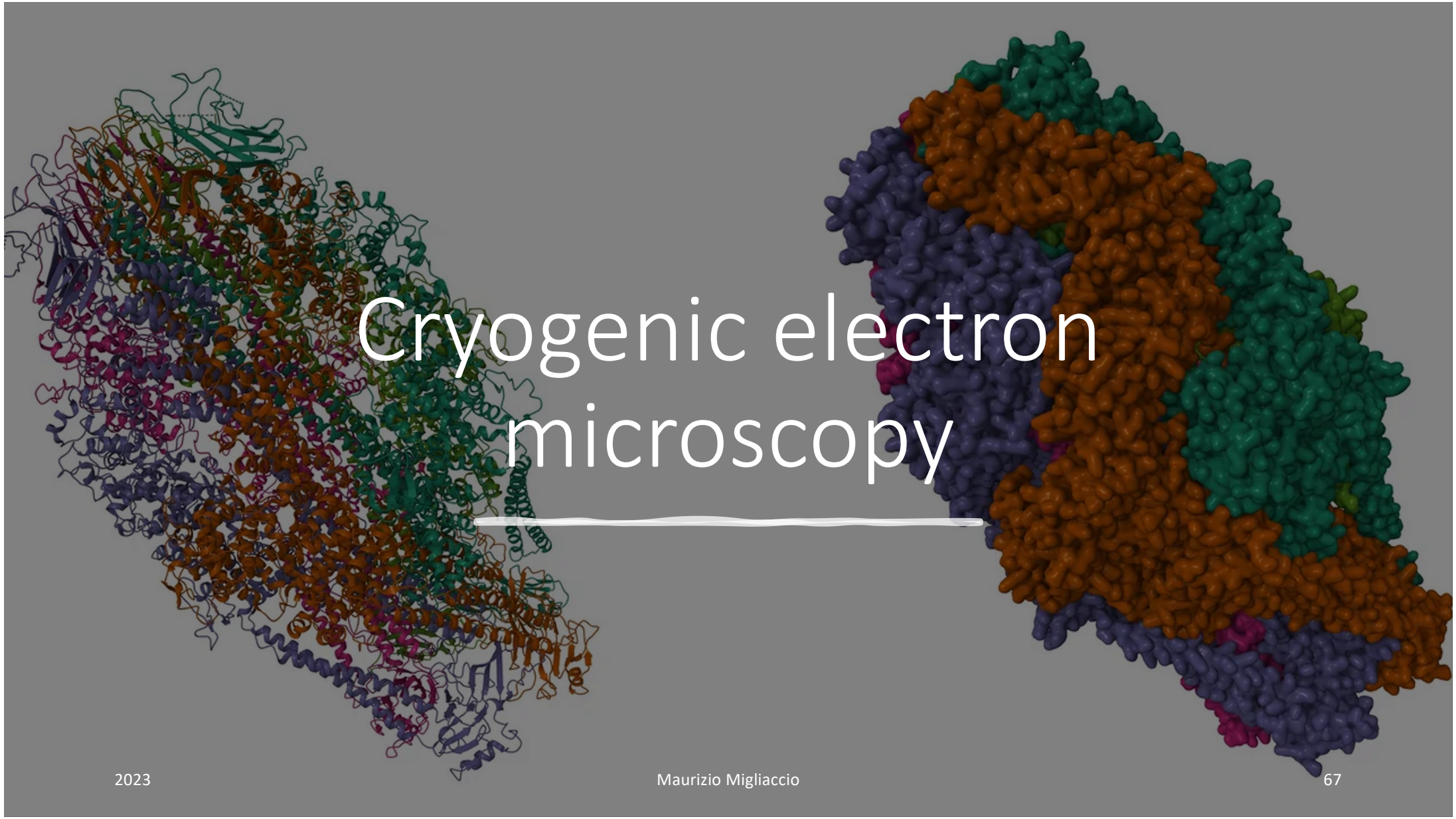


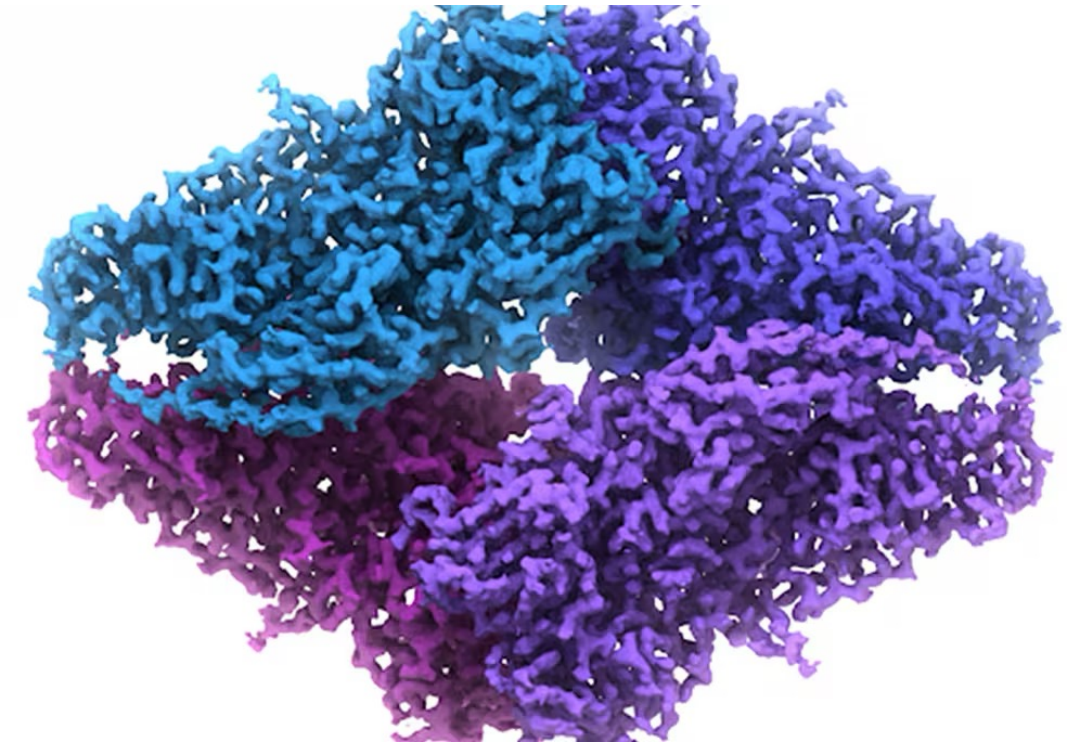
Cryogenic electron microscopy

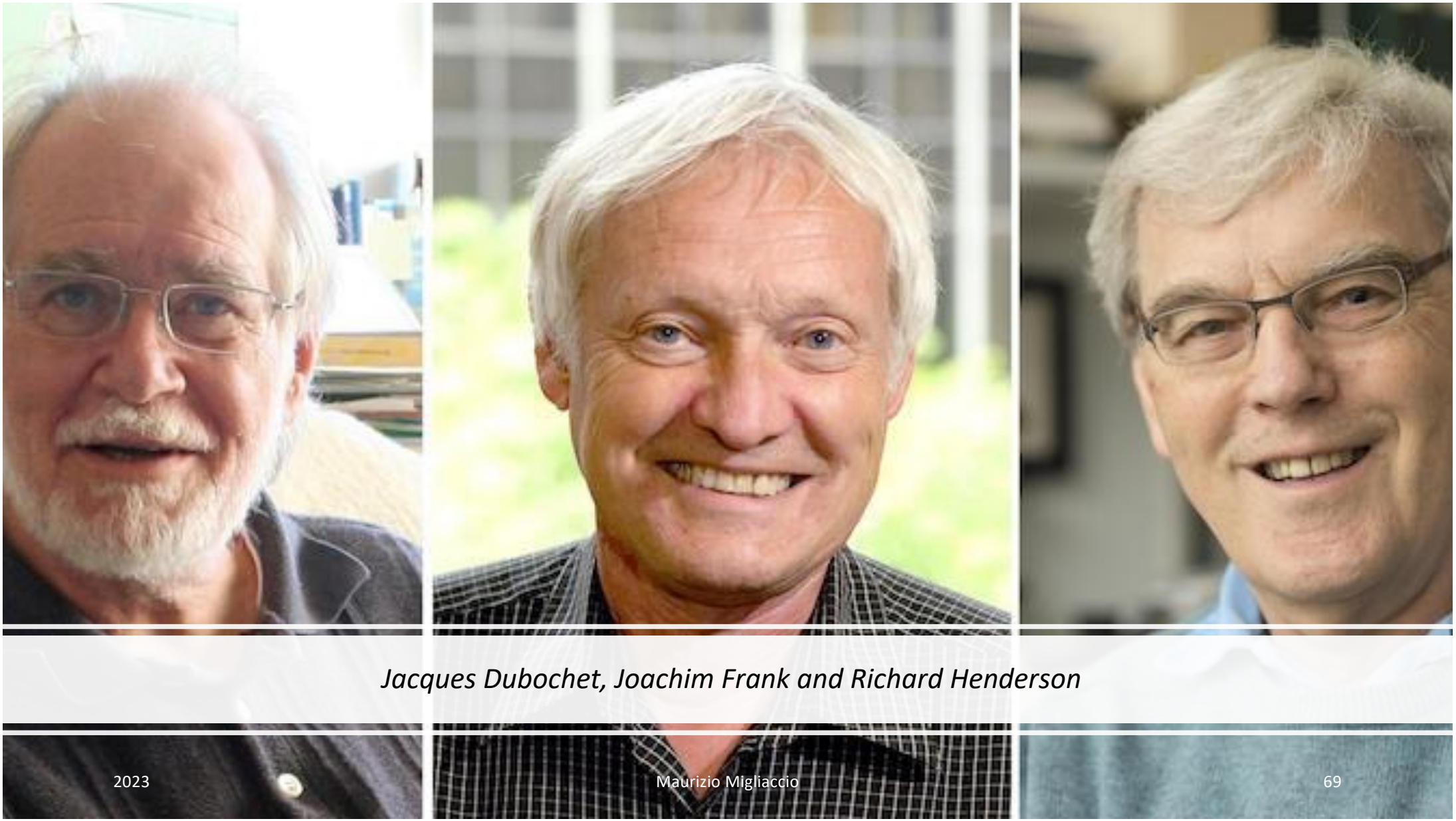
Cryo electron microscopy

The structure of a relatively small bacterial enzyme called Beta-Galactosidase seen with an instrument using cryo-electron microscopy. It has a definition of 2.2 Angstroms.

The *2017 Nobel Prize* in Chemistry was awarded to three researchers whose research led to the invention of a revolutionary microscopic imaging technique, cryo-electron microscopy.

The three researchers are Jacques Dubochet, Joachim Frank and Richard Henderson and the Nobel motivation reads "for having developed cryo-electron microscopy to determine the structures of biomolecules in high definition".





Jacques Dubochet, Joachim Frank and Richard Henderson

Cryo EM

- Cryo EM is an advanced imaging technique used to elucidate the three-dimensional (3D) structure of biological molecules and complexes at near-atomic resolution.
- In this technique, the sample is rapidly frozen to temperatures below -150 °C, trapping it in vitreous ice.
- The sample is then imaged from different angles using an electron microscope, where a beam of electrons passes through the specimen, resulting in a set of two-dimensional (2D) projections.
- Advanced computational algorithms are then used to reconstruct a 3D model of the sample from these projections.

Cryo EM

Cryo EM displays a series of important differences compared to conventional EM that makes it a powerful tool for structural biology, providing insights into previously inaccessible areas of research.

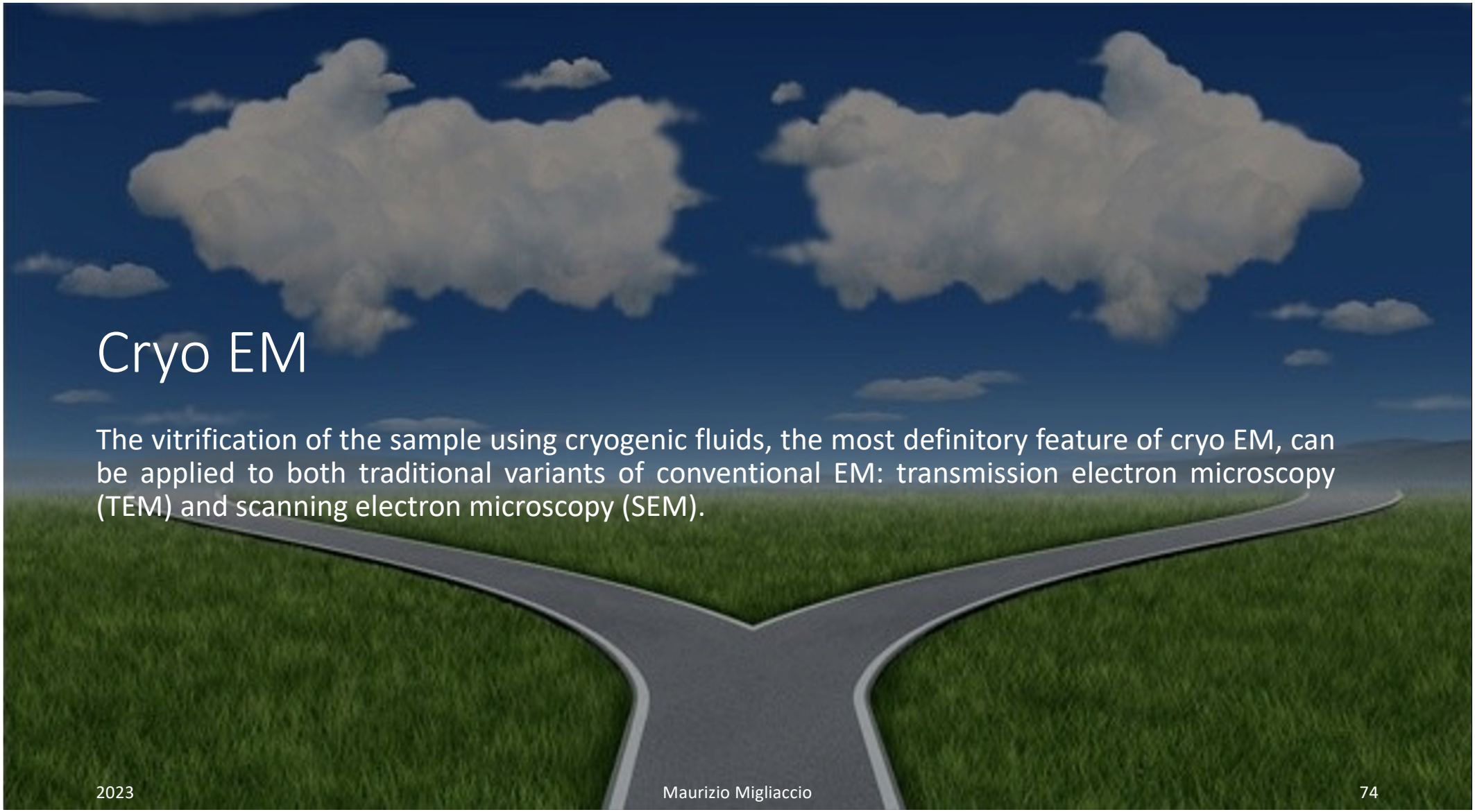
	Conventional EM	Cryo EM
Sample preparation	Involves fixation, staining, dehydration and the use of chemical compounds or polymers	Involves flash-freezing techniques (vitrification)
Sample integrity	Native structures can be altered and artifacts may appear	Native structures are well preserved
Resolution	High resolution (nm)	Higher resolution, near-atomic (Å)
Specimen types	Ideal for cellular and subcellular structures	Ideal for biomolecules and complexes
Amount of sample	Larger amounts of sample are required	Minimal amounts of biomolecules or complexes can be analyzed

Cryo EM

- The development of cryo-EM is a consequence of the limitations of conventional EM.
- Since the publication of the first 3D structure of a biological specimen obtained using EM in 1968, scientists tried to find strategies to circumvent the main problems of this technique, i.e., sample destruction caused by the high energy electron beam, alteration of the native structures and generation of undesiderable artifacts derived from the sample preparation process and low resolution when strong diffraction is present.

Cryo EM

The cryo EM era started in the early 1980s with the development of a methodology that applied a flash freezing approach that rapidly cools the samples at temperatures below the water glass-transition temperature (around -140 °C) within milliseconds.



Cryo EM

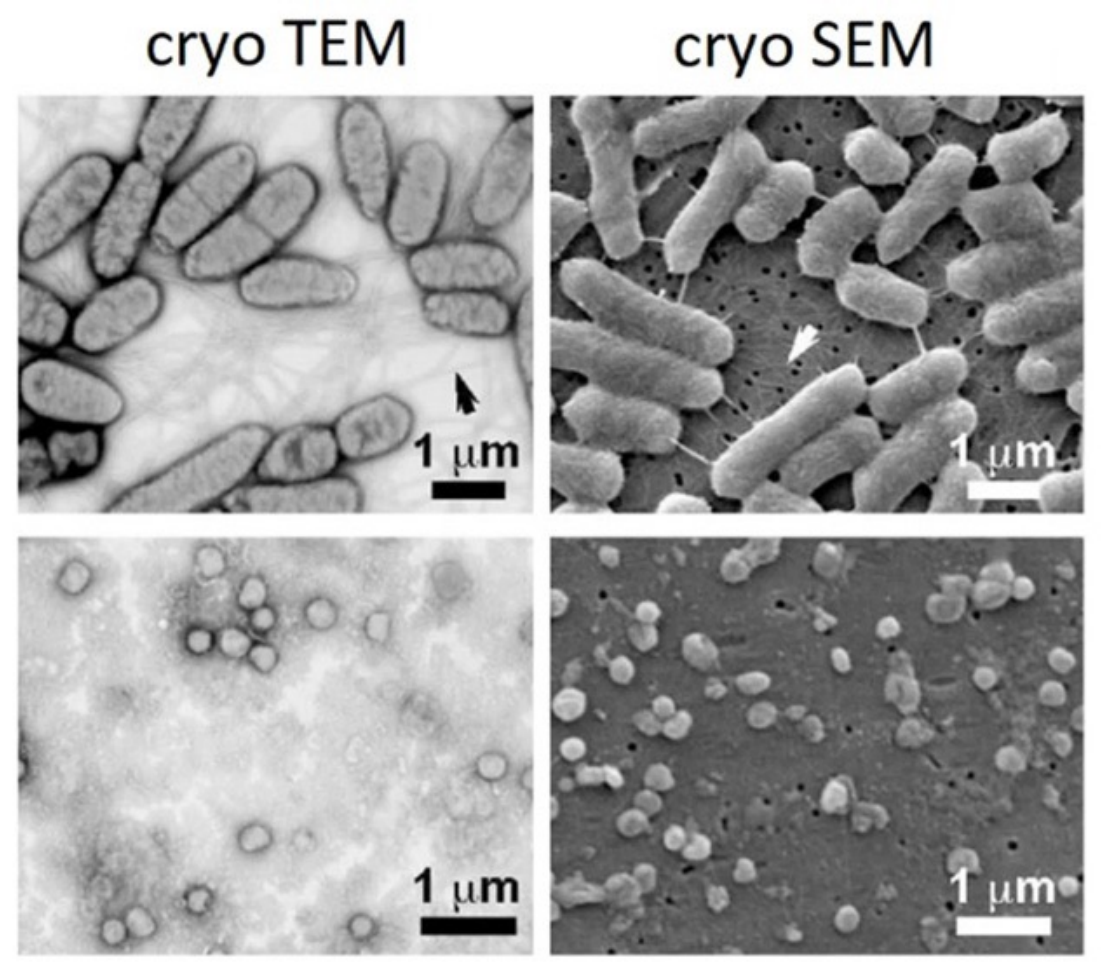
The vitrification of the sample using cryogenic fluids, the most definitory feature of cryo EM, can be applied to both traditional variants of conventional EM: transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Cryo TEM

- In this technique, the vitrified sample is loaded into a high-vacuum TEM instrument.
- Inside the TEM, an electron beam is generated and directed onto the sample. As the beam passes through the sample, it interacts with the electrons, resulting in scattering and absorption. This interaction leads to the formation of a projection image on a detector, capturing the 2D structural information of the sample.
- To obtain a 3D structure, multiple projection images are collected by tilting the sample at different angles within the microscope. These images are then processed using advanced computational algorithms to generate a 3D density map of the sample.
- This density map represents the distribution of electrons within the sample and provides insights into the atomic arrangement of the molecules.

Cryo SEM

- This technique is used to study the surface structure and topography of the vitrified samples.
- The vitrified sample is usually fractured or cleaved to expose its internal surfaces and then transferred to a SEM instrument.
- This microscope generates a focused electron beam that scans the sample's surface. As the beam interacts with the sample, secondary and backscattered electrons are emitted, which are then detected and used to create an image of the sample's surface.



Cryo EM

Examples of images obtained with cryo TEM (left) and cryo SEM (right)
of Salmonella Senftenberg (upper row) and vaccinia virus (lower row).

Credit: Adapted from Golding et al., 2016

Cryo EM

Comparison of the main features of cryo TEM and cryo SEM.

	Cryo TEM	Cryo SEM
Equipment	Transmission electron microscope	Scanning electron microscope
Electron beam	It is transmitted through the sample	It is focused on the surface
Sample thickness	Thin samples	Any thickness
Information obtained	Internal structure of the sample	Surface topography and morphology of the sample
Resolution	High resolution (near-atomic)	Lower resolution
Applications	Molecular structures and interactions. Suitable for single-particle analysis	Surface characterization. Suitable for large-scale structures or materials

Cryo ET

- Cryo electron tomography (cryo ET) is a technique that combines the principles of cryo EM and tomography, and it is especially useful to investigate cellular and subcellular 3D structures.
- This approach is based on the acquisition of a series of images taken at different tilt angles of the specimen with respect to the electron beam.
- The different planes collected are combined using computational algorithms to reconstruct the 3D structure.

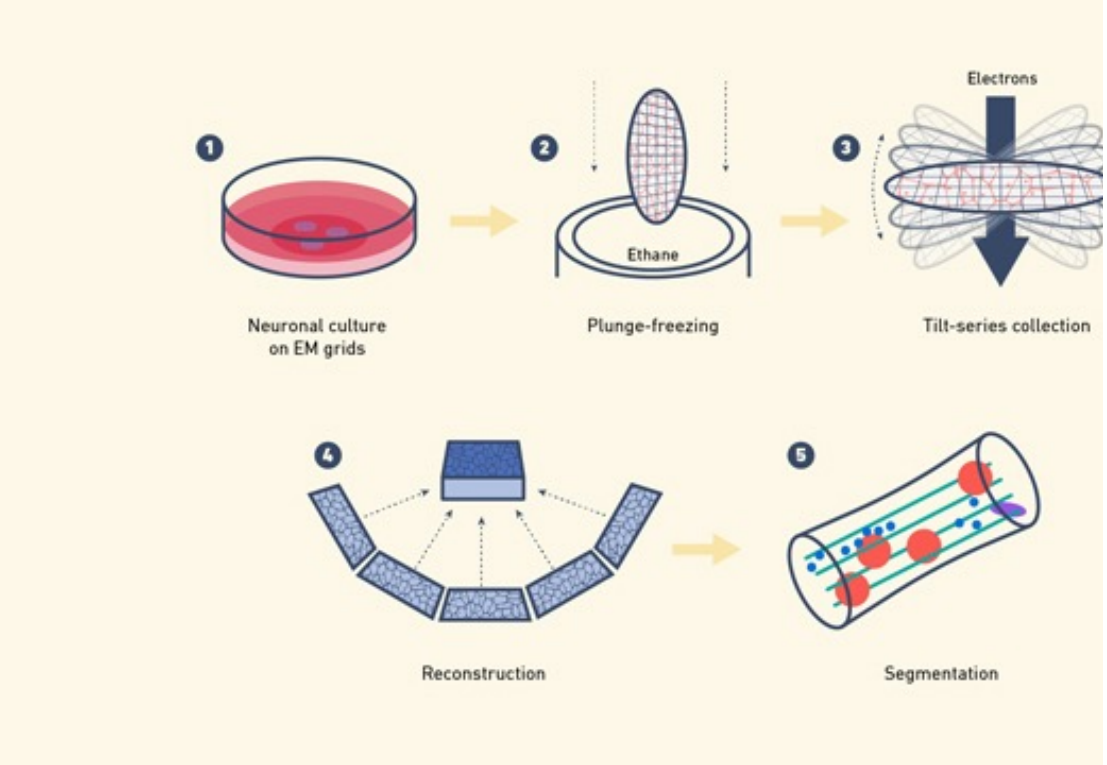
Cryo ET

The typical workflow of cryo ET is similar to that of cryo EM, including the preparation of the biological sample by isolation and preservation through vitrification.

The sample is placed onto a grid, forming a thin, even layer, and then the grid is transferred to a cryo-TEM microscope to acquire images of the sample from various angles by tilting the grid.

The images captured constitute a tomographic dataset, which contains a series of 2D projections of the sample that provide information about its structure from different orientations.

These 2D projections are aligned and corrected to minimize any misalignment or distortion, and finally, iterative algorithms or weighted back-projection is employed to reconstruct a 3D tomogram.



Cryo ET

- The advantages of cryo ET include its ability to study intact cells and tissues, preserving the spatial relationships between different cellular components.
- It provides 3D information at nanometric resolution and it is also suitable for studying dynamic processes within cells, as it allows for time-resolved imaging that capture structural changes over time.
- On the other hand, this technique also has some limitations, most of them also typical of cryo EM, such as the complex sample preparation or the necessity of using low dose electron beams to avoid sample damage, which results in lower signal-to-noise ratio.
- Additionally, the sample thickness is limited to ~ 500 nm and the range of tilt angle is limited so the information obtained is partial.

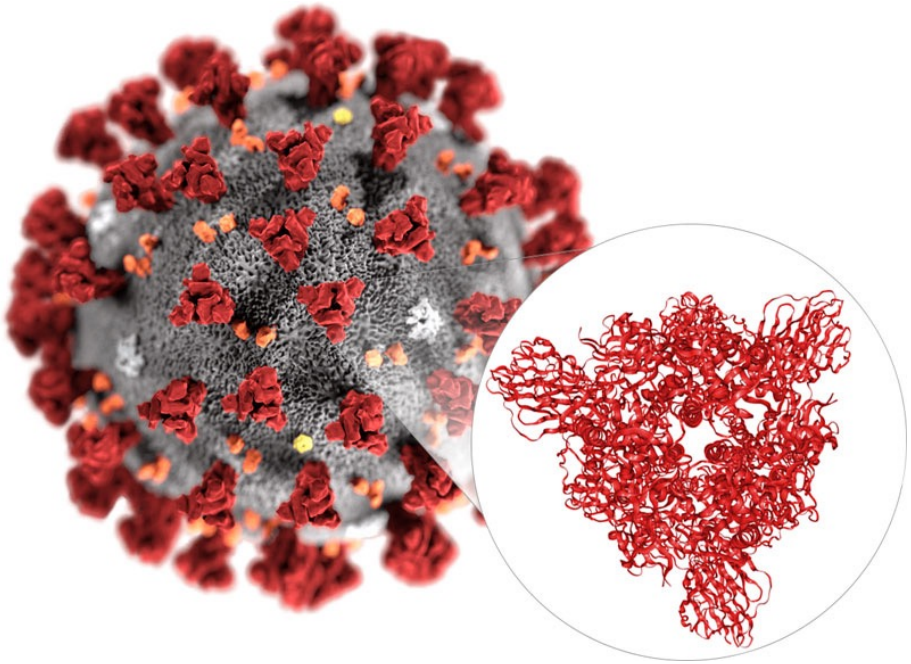
Cryo ET vs Cryo EM

- All these features make cryo ET more suitable for the study of cellular structures within their native environment, whereas cryo EM provides high-resolution imaging of individual macromolecular complexes, providing atomic-level details.
- Both techniques are often complementary in addressing different biological questions, ranging from molecular structures to cellular organization and dynamics.

Cryo EM

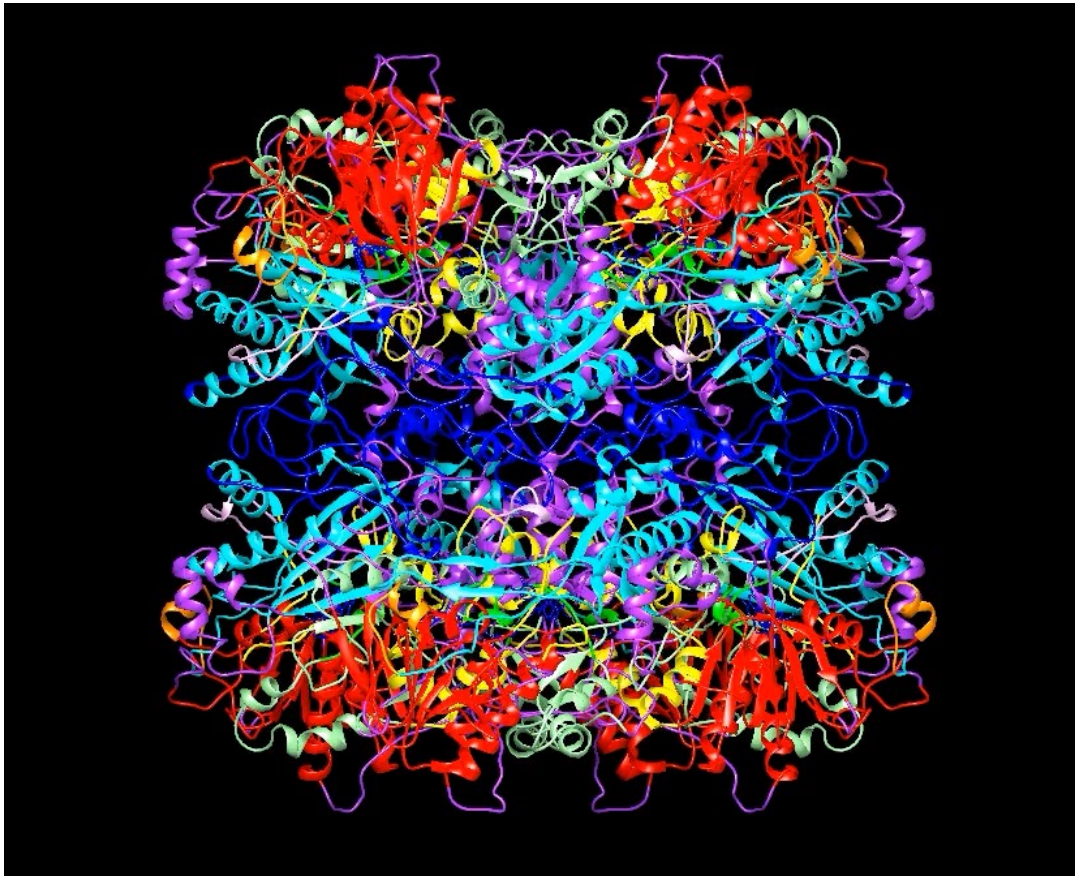
Cryo-EM has been used to generate the first 3D atomic-scale map of the coronavirus (2019-nCoV).

This was a key step towards developing a vaccine and treatments for the infection.

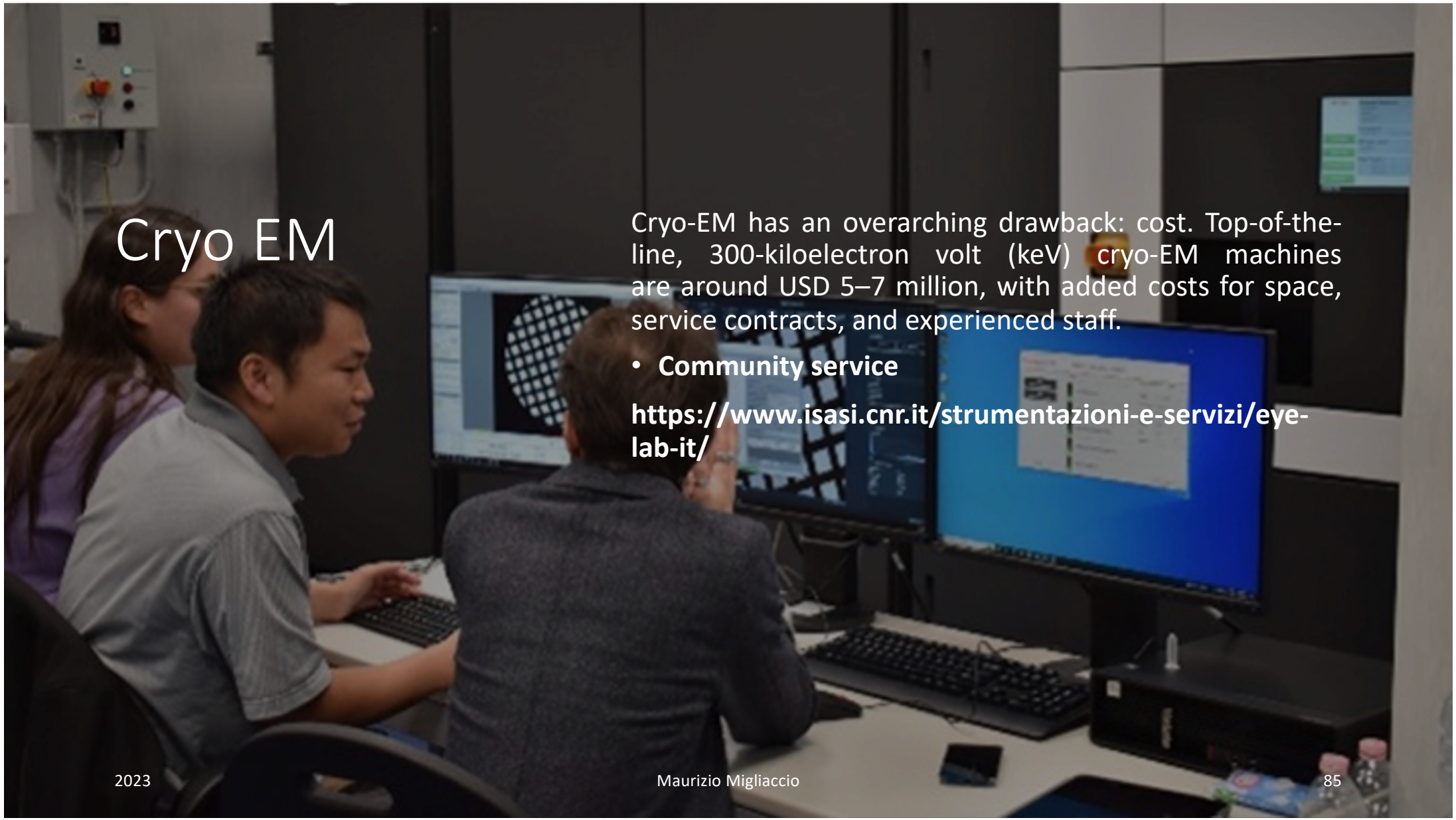


Cryo EM

Structure of alcohol oxidase from Pichia pastoris obtained by cryo-electron microscopy.



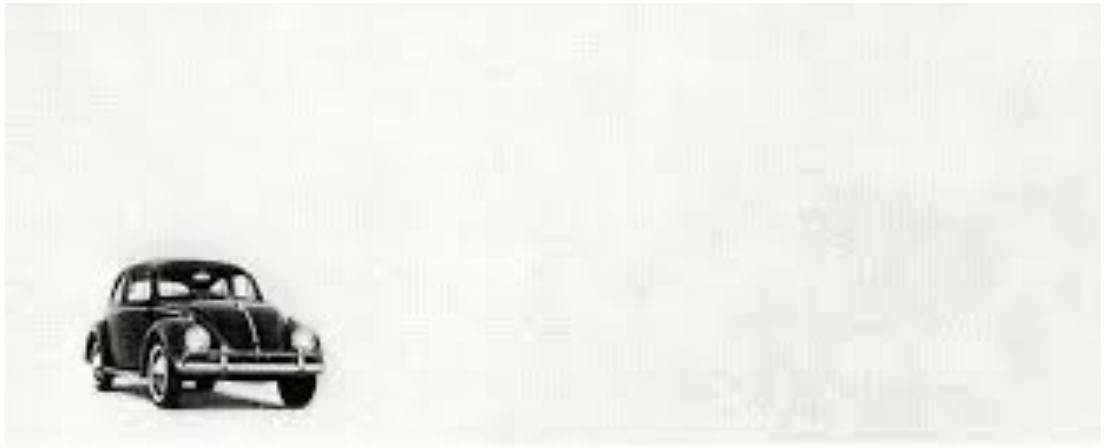
Cryo EM



Cryo-EM has an overarching drawback: cost. Top-of-the-line, 300-kiloelectron volt (keV) cryo-EM machines are around USD 5–7 million, with added costs for space, service contracts, and experienced staff.

- **Community service**

https://www.isasi.cnr.it/strumentazioni-e-servizi/eye-lab-it/



- For the person for whom small things do not exist, the great is not great.
- *Jose Ortega y Gasset* (9 May 1883 – 18 October 1955) was a Spanish philosopher and essayist.

Conclusions