Microscopy

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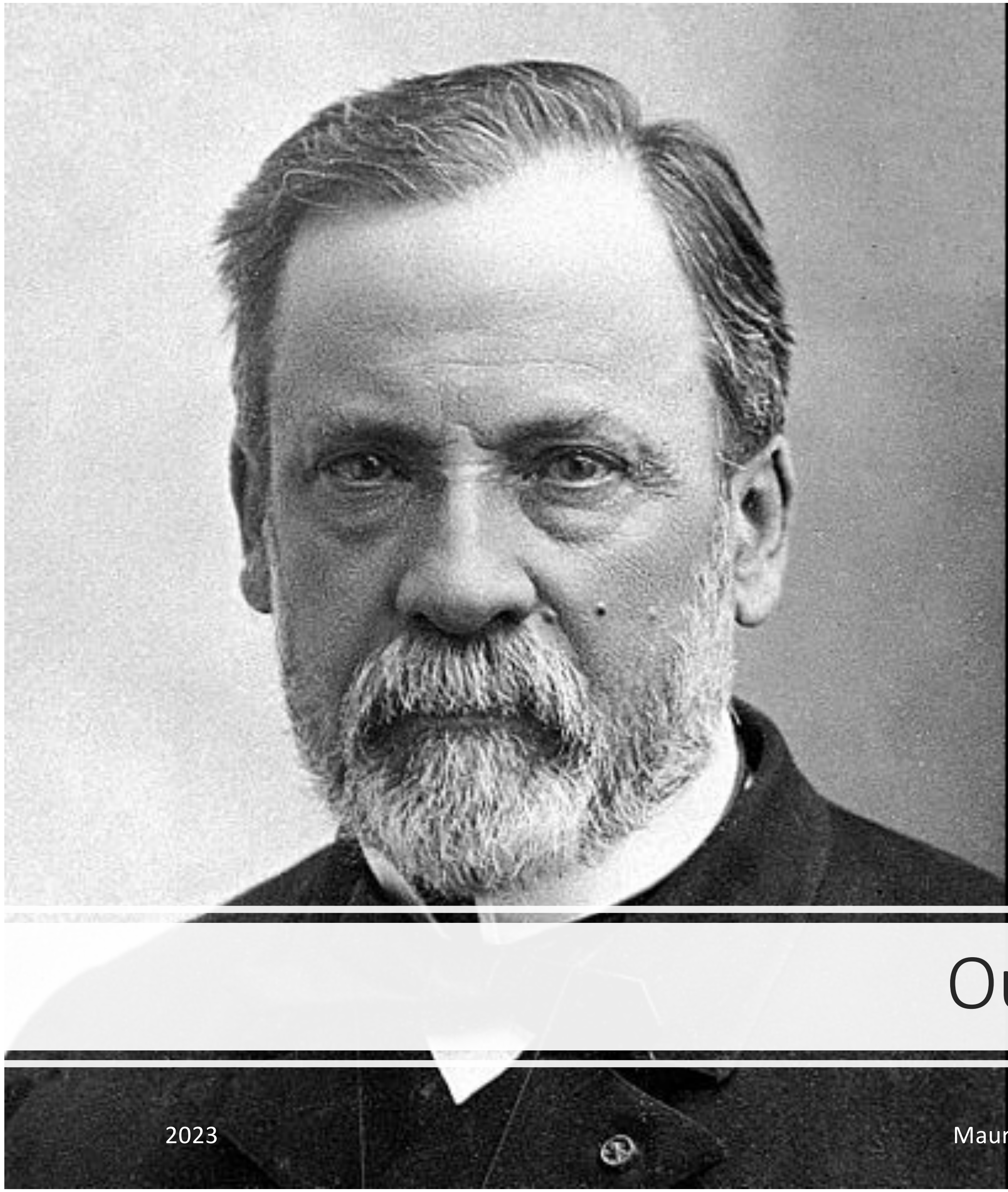
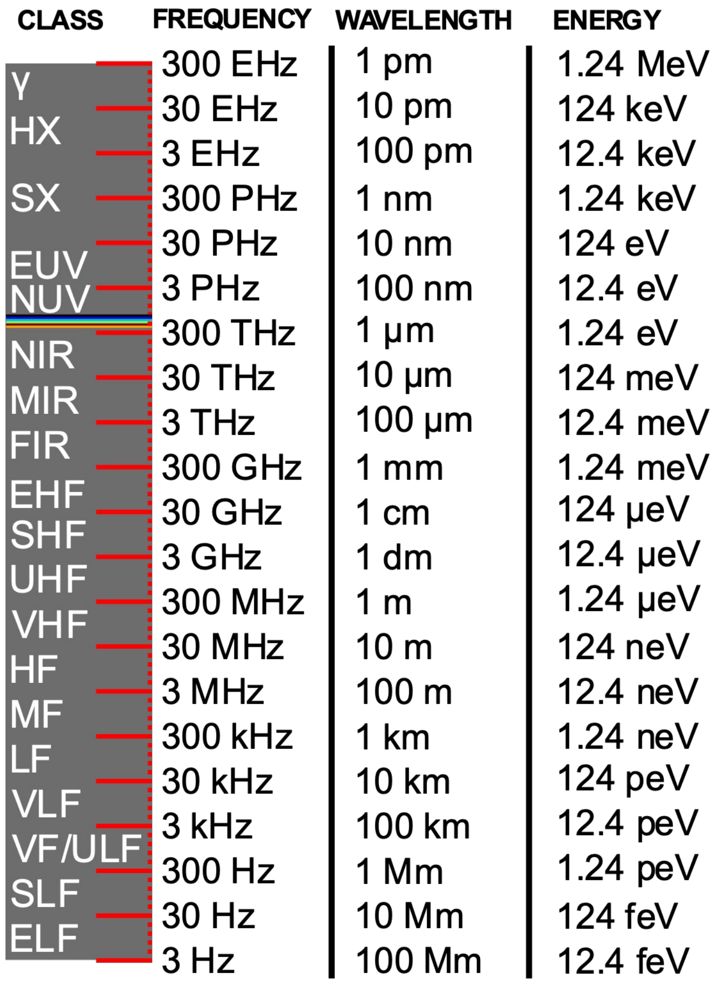


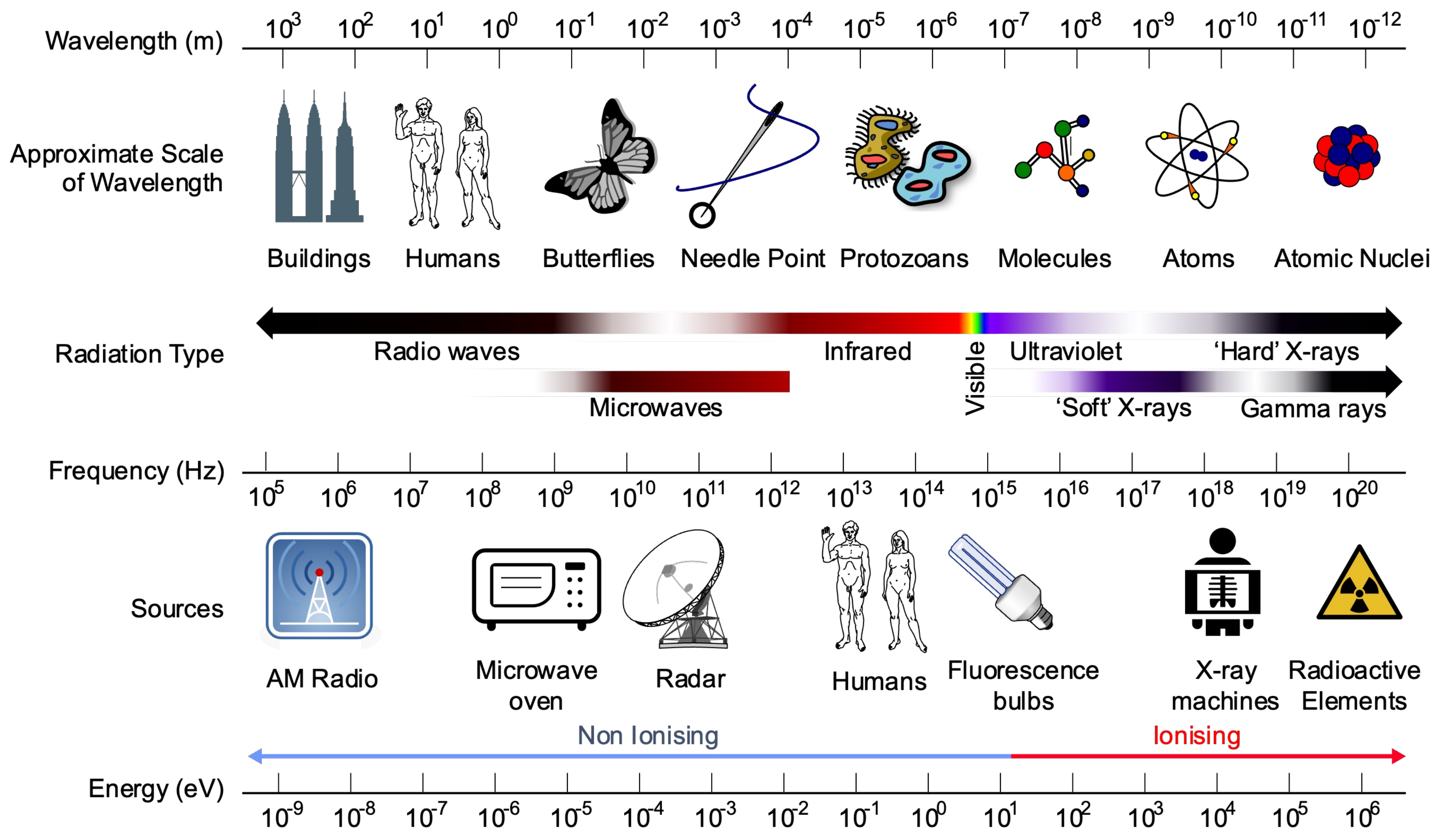
“In the fields of observation chance favors only the prepared mind.”

Outline ~ *Louis Pasteur*

Electromagnetic spectrum

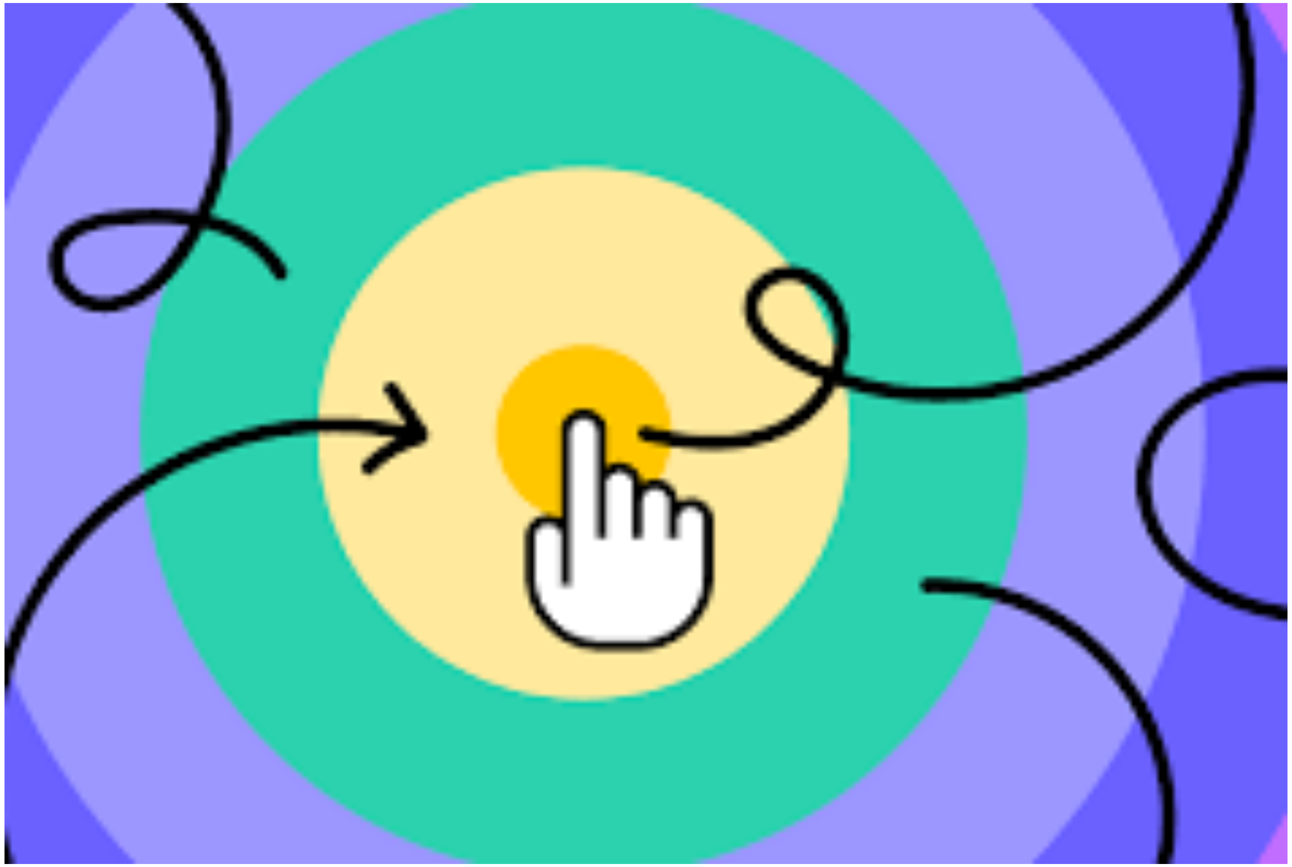
- The electromagnetic spectrum is the range
  of frequencies of electromagnetic radiation and their
  respective wavelengths and photon energies.





Wavelength (m)
10^3 10^2 10^1 10^0 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8} 10^{-9} 10^{-10} 10^{-11} 10^{-12}
Approximate Scale of Wavelength
Buildings
Humans
Butterflies
Needle Point
Protozoans
Molecules
Atoms
Atomic Nuclei
Radiation Type
Radio waves
Infrared
Visible
Ultraviolet
'Hard' X-rays
Microwaves
'Soft' X-rays
Gamma rays
Frequency (Hz)
10^5 10^6 10^7 10^8 10^9 10^{10} 10^{11} 10^{12} 10^{13} 10^{14} 10^{15} 10^{16} 10^{17} 10^{18} 10^{19} 10^{20}
Sources
AM Radio
Microwave oven
Radar
Humans
Fluorescence bulbs
X-ray machines
Radioactive Elements
Non Ionising
Ionising
Energy (eV)
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^0 10^1 10^2 10^3 10^4 10^5 10^6

Electromagnetic interaction depends on the size of the particles with respect to the wavelength. Further the physical mechanisms of interaction depends on the wavelength.



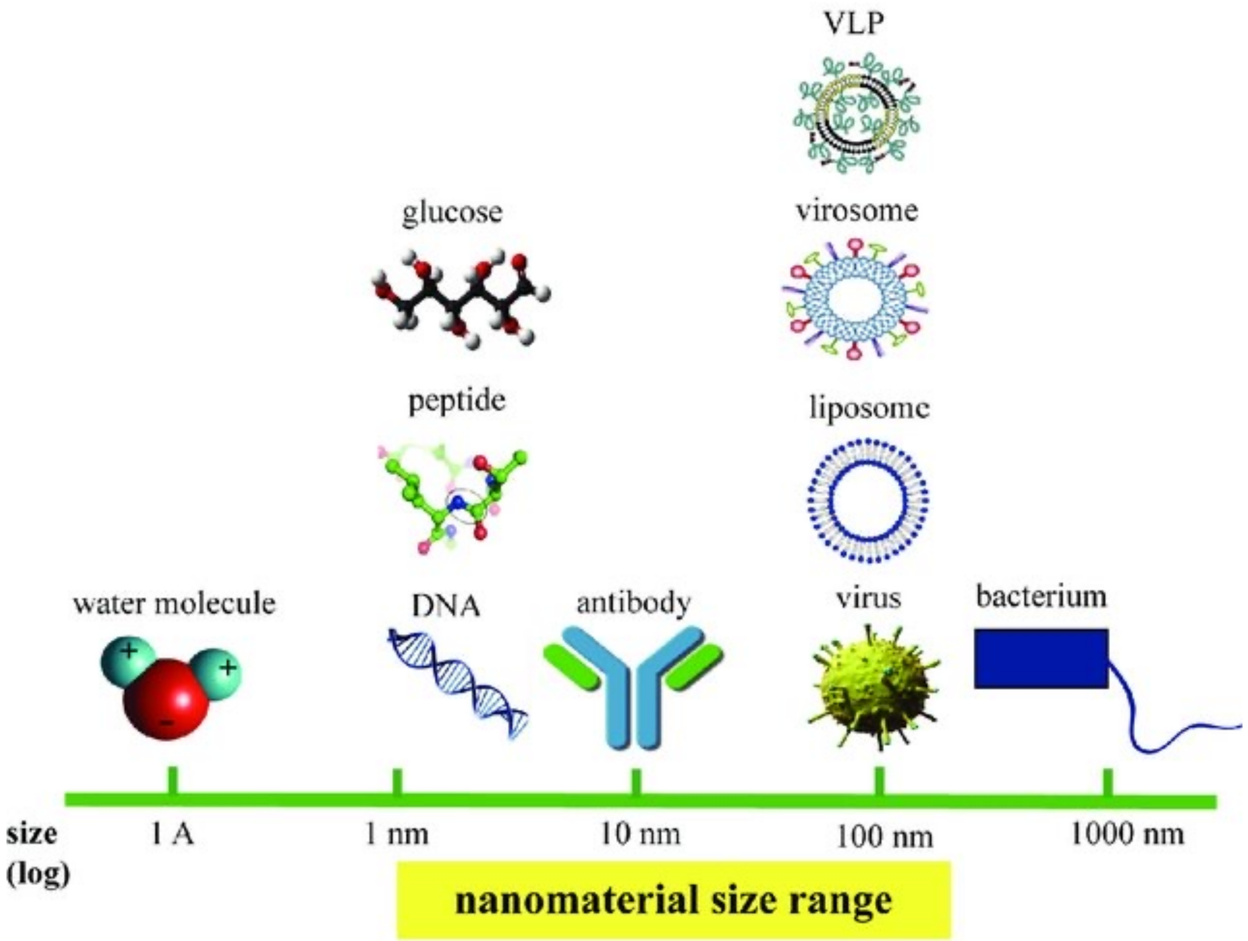
Visible band

- Only in this band of the electromagnetic spectrum (0.4 μm – 0-7μm) we can associate the concept of (true) color:
  - Violet: 0.4 - 0.446 μm
  - Blue: 0.446 - 0.500 μm
  - Green: 0.500 - 0.578 μm
  - Yellow: 0.578 - 0.592 μm
  - Orange: 0.592 - 0.620 μm
  - Red: 0.620 - 0.7 μm

Maurizio Migliaccio

Biological sizes

- Size comparison of biological substances: the size range of nanomaterials used in nanotechnology relative to the size of various biological system. VLP: virus-like particle.



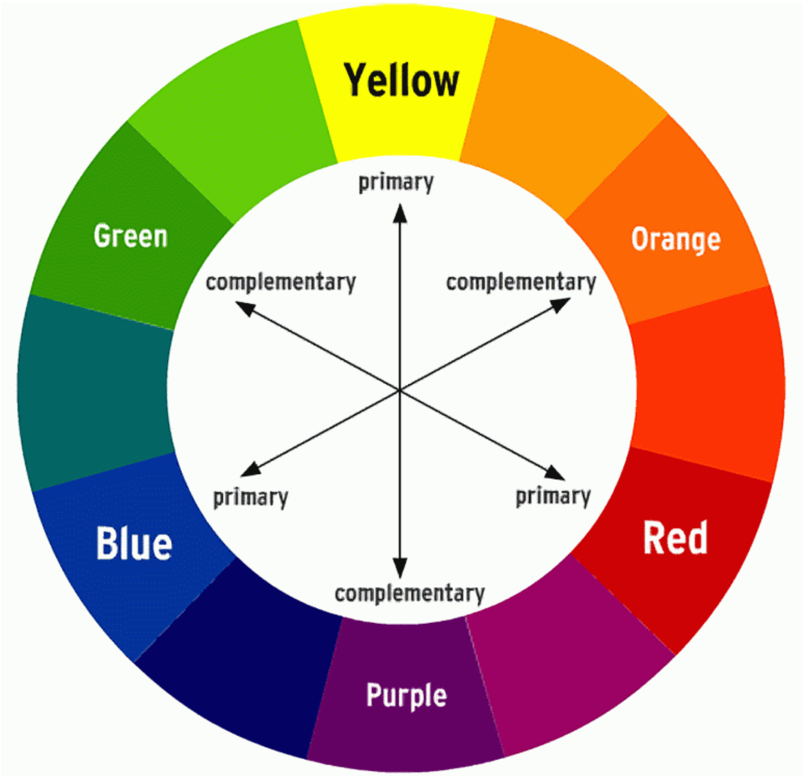
Sizes

- The ångström (Å), is a length measure not belong to the *Système International* (SI) equal to 0,1 nm or 1×10^{-10} m

MULTIPLI	Yotta	Y	$\times 10^{24}$	× 1.000.000.000.000.000.000.000.000
	Zetta	Z	$\times 10^{21}$	× 1.000.000.000.000.000.000.000
	Esa, Exa	E	$\times 10^{18}$	× 1.000.000.000.000.000.000
	Peta	P	$\times 10^{15}$	× 1.000.000.000.000.000.
	Tera	T	$\times 10^{12}$	× 1.000.000.000.000.
	Giga	G	$\times 10^{9}$	× 1.000.000.000.
	Mega	M	$\times 10^{6}$	× 1.000.000.
	Chilo	K	$\times 10^{3}$	× 1.000
	Etto	h	$\times 10^{2}$	× 100
	Deca	da	$\times 10^{1}$	× 10
	UNITÀ		$\boldsymbol{\times 10^{0}}$	**× 1**
SOTTOMULTIPLI	Deci	d	$\times 10^{-10}$	: 10
	Centi	c	$\times 10^{-2}$	: 100
	Milli	m	$\times 10^{-3}$	: 1.000
	Micro	µ	$\times 10^{-6}$	: 1.000.000
	Nano	n	$\times 10^{-9}$	: 1.000.000.000
	Pico	p	$\times 10^{-12}$	: 1.000.000.000.000
	Femto	f	$\times 10^{-15}$	: 1.000.000.000.000.000
	Atto	a	$\times 10^{-18}$	: 1.000.000.000.000.000.000
	Zepto	z	$\times 10^{-21}$	: 1.000.000.000.000.000.000.000
	Yocto	y	$\times 10^{-24}$	: 1.000.000.000.000.000.000.000.000

Interaction

- A body can absorb energy by an electromagnetic wave because of the molecular/atomic energy variations.
- Absorption depends not only on the specific body but also on the e.m. wavelength. Any body is characterized by its peculiar absorption spectra.
- Any colored body is characterized by some peaks that are located at complementary color.



Interaction

- Biological molecules do not (or marginally) absorb visible e.m. waves.
- Therefore, the light pass through unchanged but for a delay that is hard to be estimated. Perception on light amplitude.
- This is untrue for UV waves.

Generalities

- Microscopy is the technical field of using microscopes to view objects and areas of objects that cannot be seen with the naked eye.
- Microscopy can be: optical, electron, and scanning probe microscopy, along with the X-ray microscopy.
- The optical microscope, also referred to as a light microscope, is a type of microscope that commonly uses visible light and a system of lenses to generate magnified images of small objects.

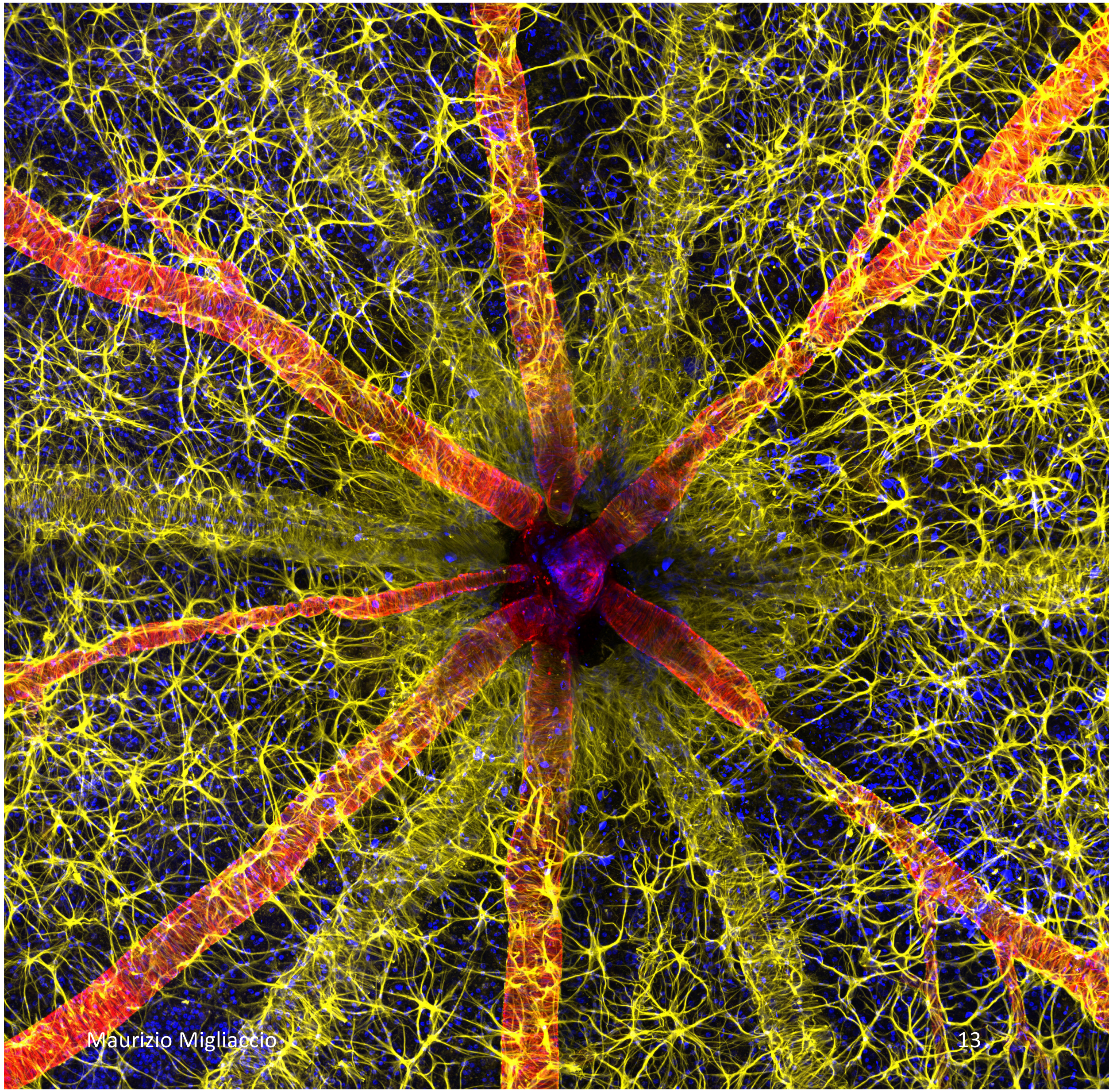
Generalities

- According to the Oxford English Dictionary a microscope is ”an instrument magnifying objects by means of lenses so as to reveal details invisible to the naked eye”.
- Thus, basically a microscope forms a magnified image of an object.
- The first microscopes used visible light.

SMALL WORLD

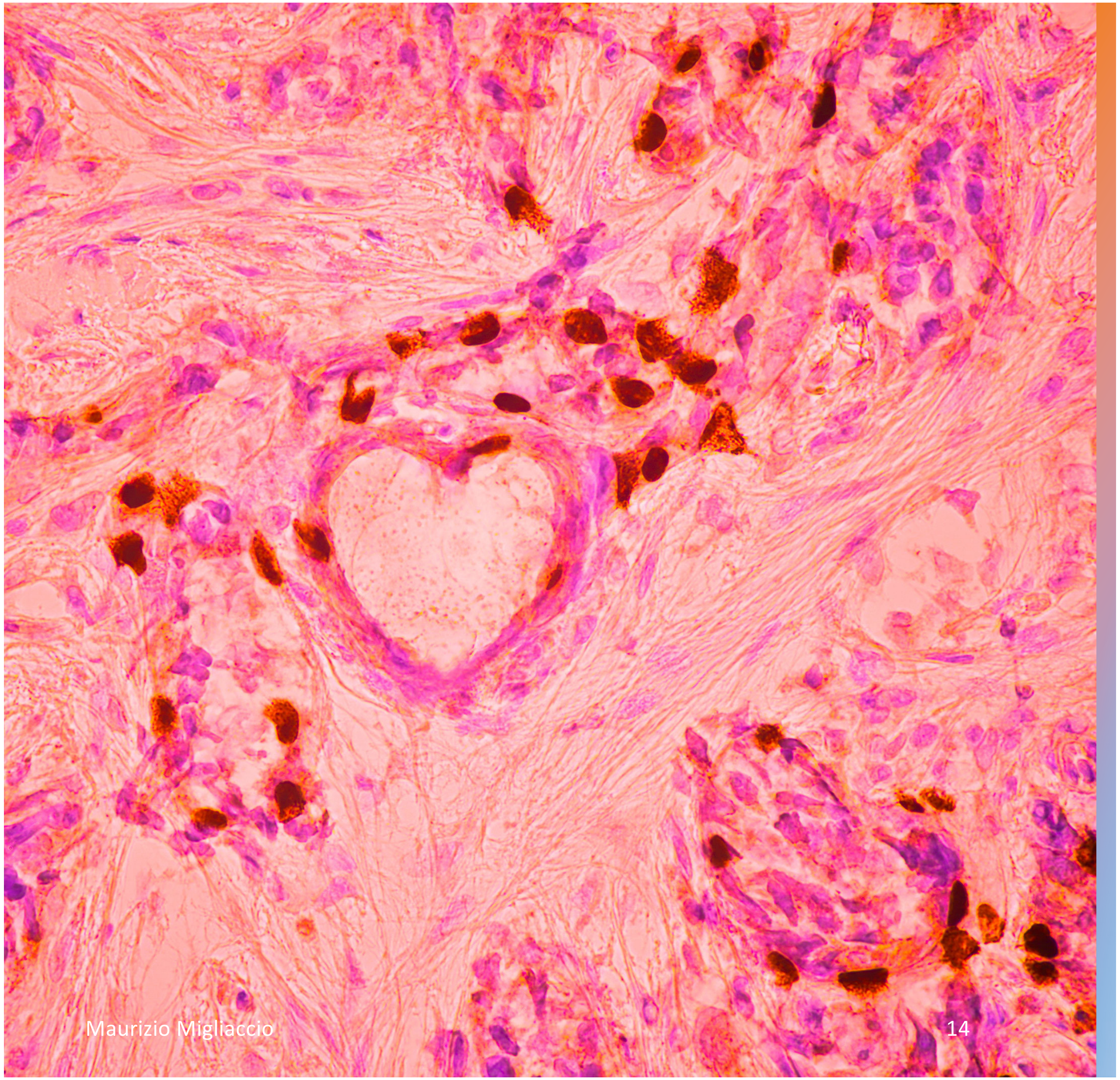
Nikon Small World

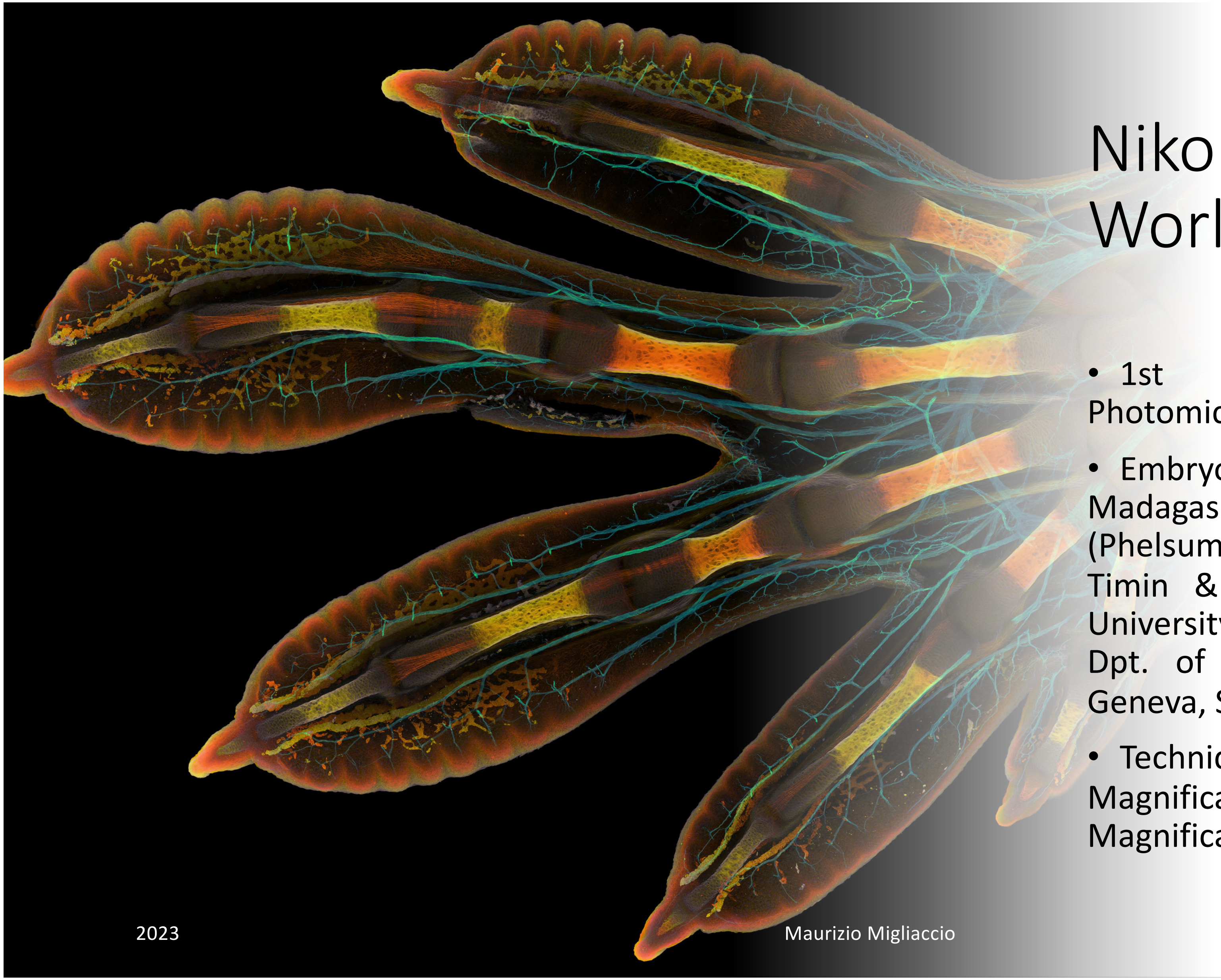
- *Nikon's Small World is regarded as the leading forum for showcasing the beauty and complexity of life as seen through the light microscope.*
- 1st Place 2023 Photomicrography Competition.
- Rodent optic nerve head showing astrocytes (yellow), contractile proteins (red) and retinal vasculature (green) by Hassanain Qambari & Jayden Dickson @ Lions Eye Institute Department of Physiology & Pharmacology Perth, Western Australia, Australia.
- Technique: Confocal, Fluorescence, Image Stacking, Magnification: 20X (Objective Lens Magnification)



Nikon Small World

- 3rd Place 2023 Photomicrography Competition.
- Breast cancer cells by Malgorzata Lisowska @ Independent Value Based Healthcare Consultant Warsaw, Mazowieckie, Poland.
- Technique: Brightfield, Image Stacking, Magnification: 40X (Objective Lens Magnification).





Nikon Small World

- 1st Place 2022 Photomicrography Competition.
- Embryonic hand of a Madagascar giant day gecko (Phelsuma grandis) by Grigorii Timin & Michel Milinkovitch @ University of Geneva Dpt. of Genetics and Evolution Geneva, Switzerland.
- Technique: Confocal, Magnification: 63X (Objective Lens Magnification).

Nikon Small World

1st Place 2013 Photomicrography Competition

Chaetoceros debilis (marine diatom), a colonial plankton organism by Wim van Egmond @ Micropolitan Museum Berkel en Rodenrijs, Zuid-Holland, The Netherlands.

Technique: Differential Interference Contrast, Image Stacking, Magnification: 250x.

Generalities

- Nowadays there are many different types of microscope. Our understanding of a microscope must therefore be generalized in the following ways.
- The microscope may not be a conventional imaging system using lenses or mirrors. For example, it may be a scanning imaging system. A confocal system is a combination of a conventional and a scanning system. Another important category of microscopes is that of probe micro- scopes, including near-field optical microscopes. Some probe microscopes, for example the atomic force microscope (AFM) do not even seem at first glance to rely on the direct use of radiation.

Generalities

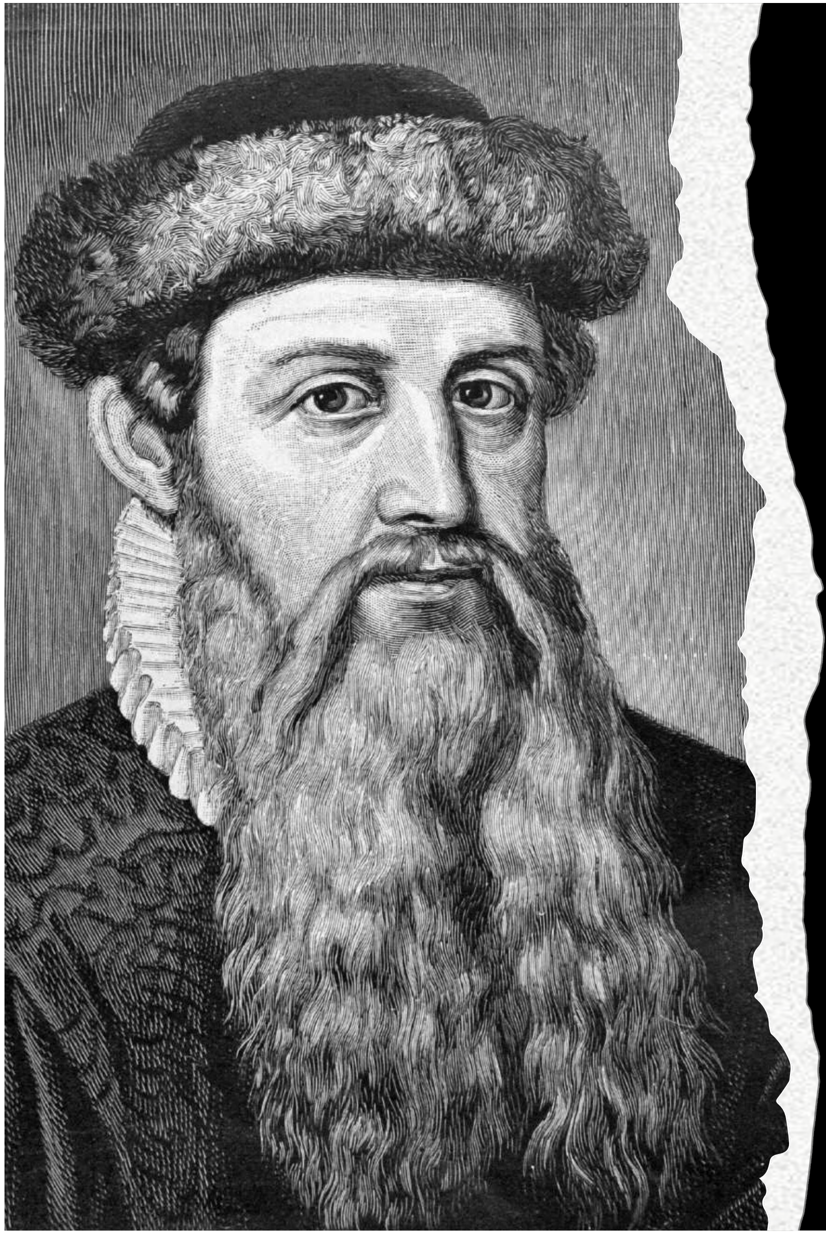
- The microscope may not use visible light, but other electromagnetic radiation, or even other forms of radiation.
- The image can be formed using a variety of different contrast mechanisms. Some of these, such as phase contrast, are designed to image particular optical properties of the sample. Others image the generation of a form of radiation when the object is stimulated by another form of radiation. In particular, in principle, virtually any form of spectroscopy can be the basis of building up an image by measuring the spatial variations in the signal.

First steps in microscopy

- *Spectacles*
- Within just a few short years in Tuscany, Italy, two men claimed to have independently invented spectacles. The evidence? Their tombstones! One, Salvano d'Aramento degli Amati died in 1284 in Florence and claimed to have kept the process secret. The other, Alessandro della Spina died in 1317 and claimed to have revealed his process. Pisa and Florence are but a short gallop away. Coincidence? You decide.
- In any event, a local monk, Girodina da Rivalta gave a sermon in 1306 in which he enthusiastically endorsed spectacles as a terrific invention and in passing, indicated that they had been in use for about 20 years. Finally, in 1289, another local from the Popozo family bemoaned that "I am so debilitated by age that without the glasses known as spectacles, I would no longer be able to read or write."
-

First steps in microscopy

- *Telescopes*
- At about the same time, it appears that lenses were being used in early telescopes. In the 13th century, the Englishman, Roger Bacon (see aside in his observatory in Oxford) discusses them at length.
- No one is quite certain about the lifespan of Roger Bacon. He was born in Ilchester, Somerset, in 1220 or thereabouts, and lived until about 1292.
- In a scientific paper written in 1260, he made an incredibly prophetic statement. Bacon said that the magnifying power of lenses could be used in an instrument we now know as a telescope, and he described what might be seen through one. “A small army may appear a very great one,” he wrote, “and Man will be able to study the Moon and the stars in great detail.” It was not until 1608, however, that a Dutchman, Hans Lippershey, produced the first efficient telescope.
- https://www.lookandlearn.com/blog/12864/roger-bacon-the-scientist-who-foresaw-the-telescope/



Johannes Gutenberg

- Johannes Gensfleisch zur Laden zum Gutenberg (c. 1393–1406 – 3 February 1468) was a German inventor and craftsman who introduced letterpress printing to Europe with his movable-type printing press.
- Though movable type was already in use in East Asia, Gutenberg invented the printing press, which later spread across the world.
- His work led to an information revolution and the unprecedented mass-spread of literature throughout Europe. It also had a direct impact on the development of the Renaissance, Reformation, and humanist movements, as all of them have been described as "unthinkable" without Gutenberg's invention.

First steps in microscopy

- *Telescopes*
- As a result, Thomas Digges' work on the telescope in England in the mid-16th century and Hans Lippershey's work which included applying for a telescope patent were transmitted to others, including no less a genius than Galileo.
- Galileo Galilei presented his first telescope – 25 august 1609.

First steps in microscopy

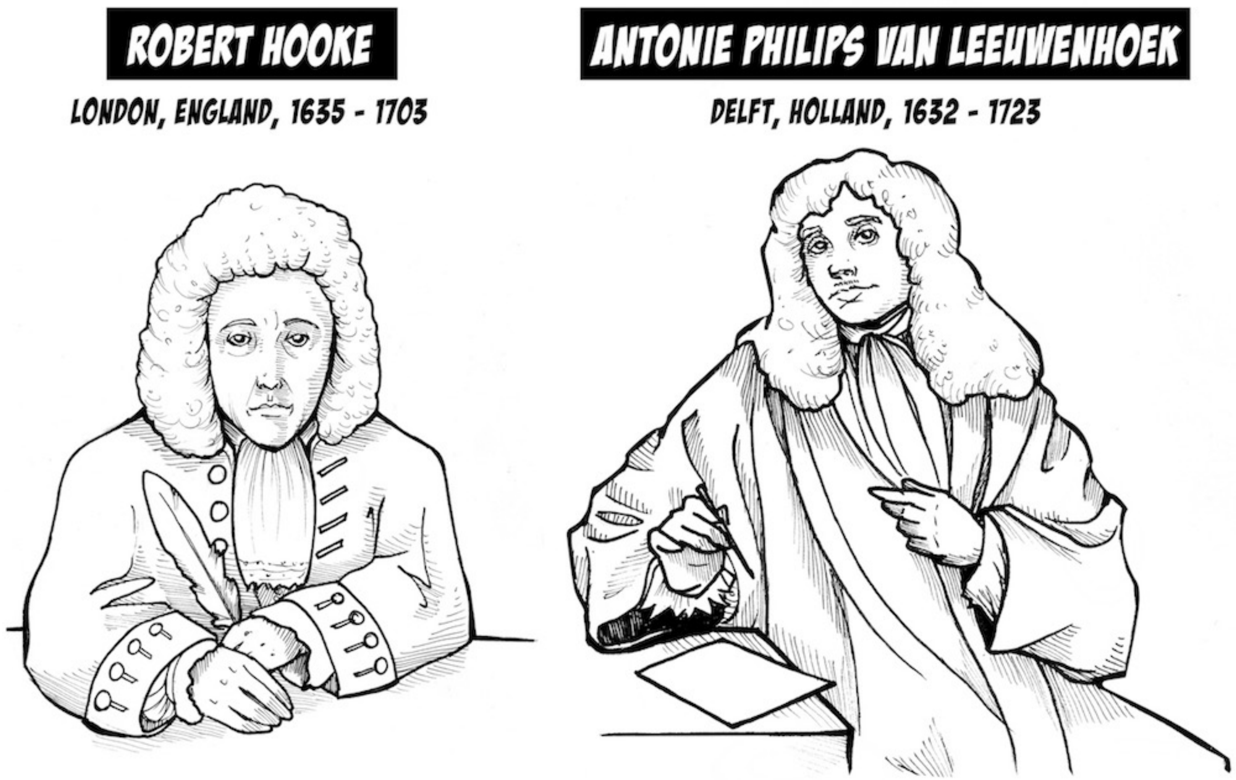
- *Telescopes*
- Hans Lipperhey (c. 1570 – buried 29 September 1619), also known as Johann Lippershey or Lippershey, was a German-Dutch spectacle-maker.
- He is commonly associated with the invention of the telescope, because he was the first one who tried to obtain a patent for it.
- It is, however, unclear if he was the first one to build a telescope.

First steps in microscopy

- Zacharias Janssen (1585 – pre-1632) was a Dutch spectacle-maker.
- He experienced the use of several lenses in a tube and were amazed to see that the object at the end of the tube was magnified significantly beyond the capability of a magnifying glass.
- He invented the compound microscope (controversial).
- That is to say, he discovered that an image magnified by a single lens can be further magnified by a second or more lenses.

First steps in microscopy

Then, in the mid 17th century, an Englishman, **Robert Hooke** and a Dutchman, **Anthonie Van Leeuwenhoek** took the microscope to new levels.

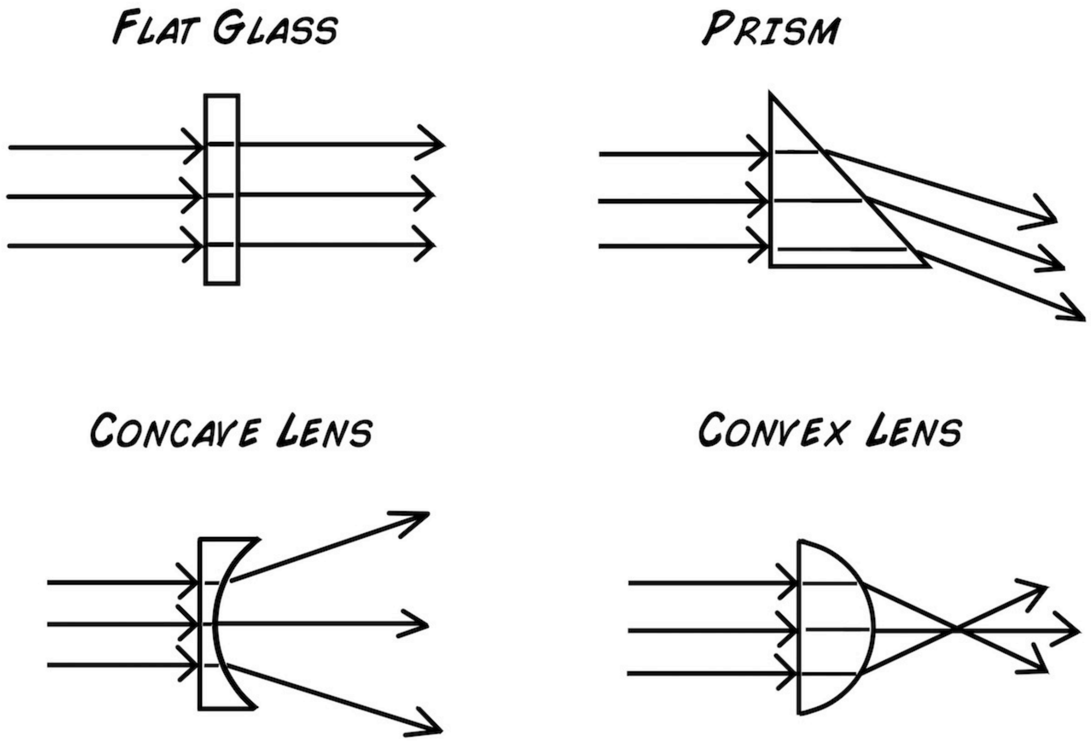


First principles

A lens has three properties, it is clear, it is curved, and it bends light.

The bending of the light is the key property that allows microscopes to magnify images.

Light bends when it enters or exits transparent material at an angle, and the curved form of a lens allows the bending to either "diverge out" or "converge in" depending on the shape of the lens.



First principles

The bending property is actually due to the speed of light.

We may think of the speed of light as a constant that can never be surpassed, but light actually propagates at different velocities depending on the material in which it is passing through.

Because of this difference in speed of light between two materials, and given light's peculiarities, when a ray of light, traveling in vacuum or air, encounters a new material, the angle will change so that light "spends less time" in the material. This level of bending is defined as an "index of refraction."

$$\text{INDEX OF REFRACTION “n”} = \frac{\text{VELOCITY OF LIGHT IN VACUUM “c”}}{\text{VELOCITY OF LIGHT IN MEDIUM “v”}}$$

$$\text{OR} \quad n = \frac{c}{v}$$

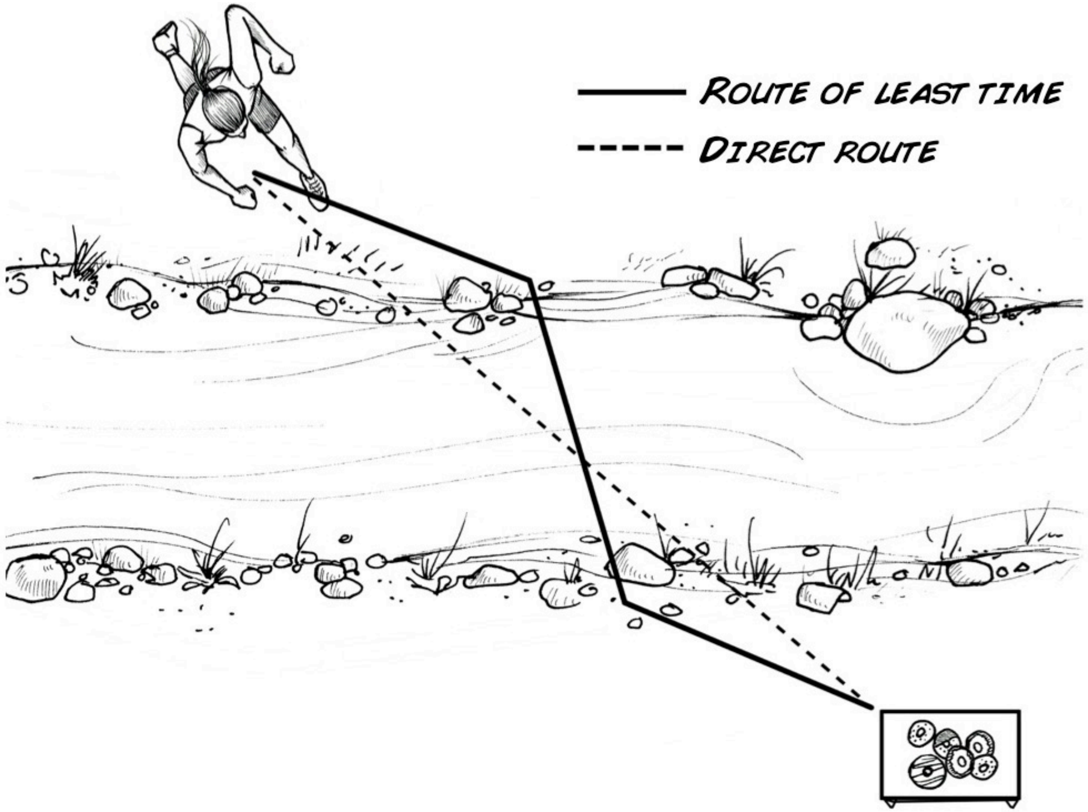
First principles

Conceptually, we can think of a famous example where a runner must cross a stream to reach a table of donuts.

The direct route is actually slower, since running in water reduces the runner's speed.

Thus, the runner actually takes the "least time route," in which she changes the angles where she enters the water and exits the water to reach the donuts in the fastest time possible.

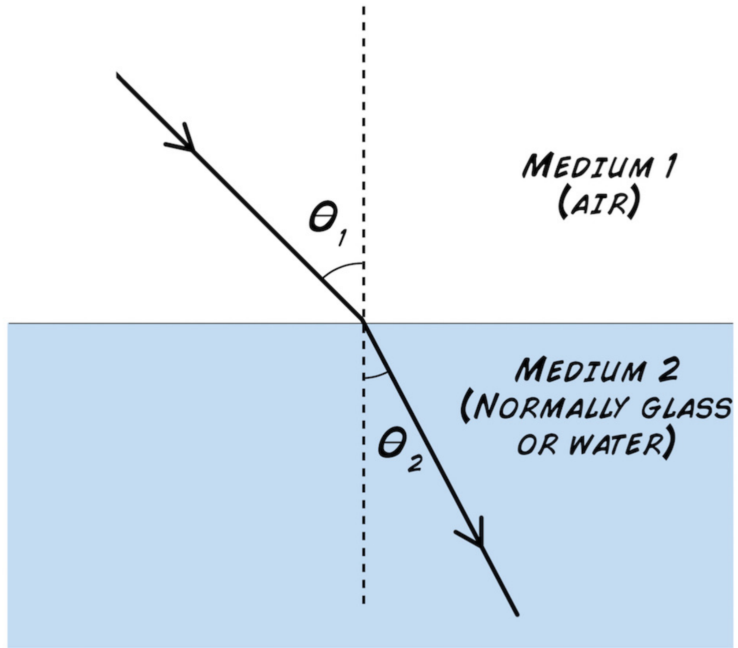
This "time minimization" serves as an analogy as to why light bends when entering a material.



First principles

Mathematically, the bending, the index of refraction, is expressed as aside (Snell’s law).

Higher a material's index of refraction, the more the light ray bends.



$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

WHERE:

n_1 : INDEX OF REFRACTION OF THE MEDIUM 1

n_2 : INDEX OF REFRACTION OF THE MEDIUM 2

First principles

SUBSTANCE	INDEX OF REFRACTION
AIR/VACUUM	1.00
WATER	1.33
ALCOHOL	1.36
VEGETABLE OIL	1.47
OPTICAL (SODA-LIME) GLASS	1.52
AMBER	1.55
CRYSTAL (LEAD) GLASS	1.75
CUBIC ZIRCONIA	2.15
DIAMOND	2.42

First principles

This mathematical relationship of the changing angles between two materials was first derived geometrically by the Persian/Baghdad scholar Ibn Sahl in the 10th century. Sahl was interested in the geometry of "burning mirrors and lenses" that can converge light rays from the sun to allow localized increases in temperature and flames.

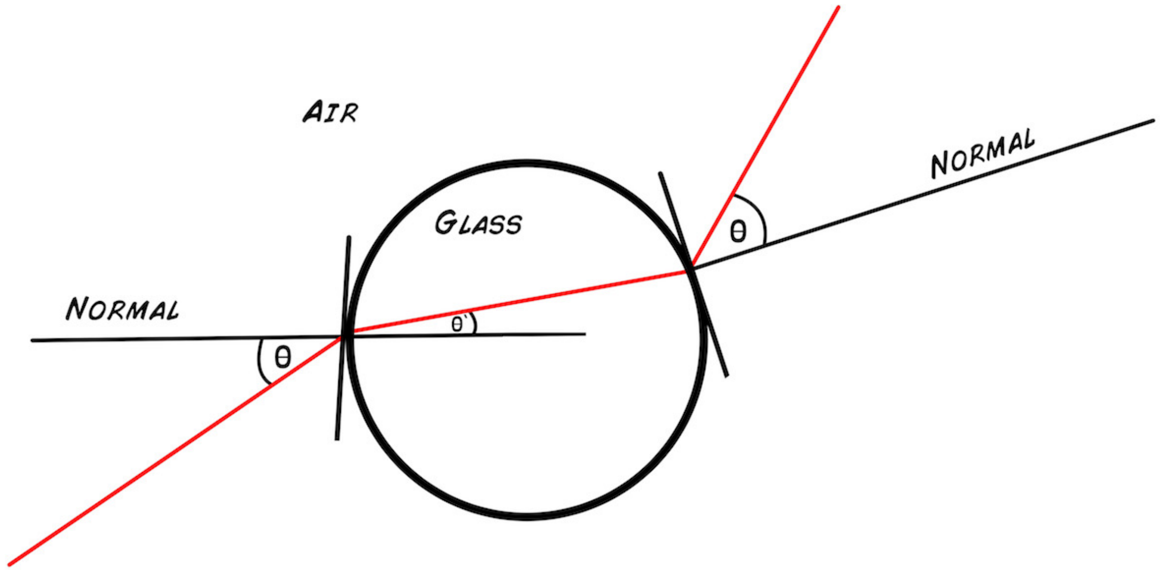
The law was then independently discovered again by Willebrord Snellius in Leiden in Holland in the early 17th century. Science history recognizes it the equation above as Snell's law, though it was known during the Islamic Golden age by Ibn Sahl and the famous optics theorist Ibn al-Haytham.

First principles

With just this simple index of refraction equation, you can calculate how lenses behave.

Remember that a lens needs be curved. With this curvature, you can cause light rays to diverge or converge.

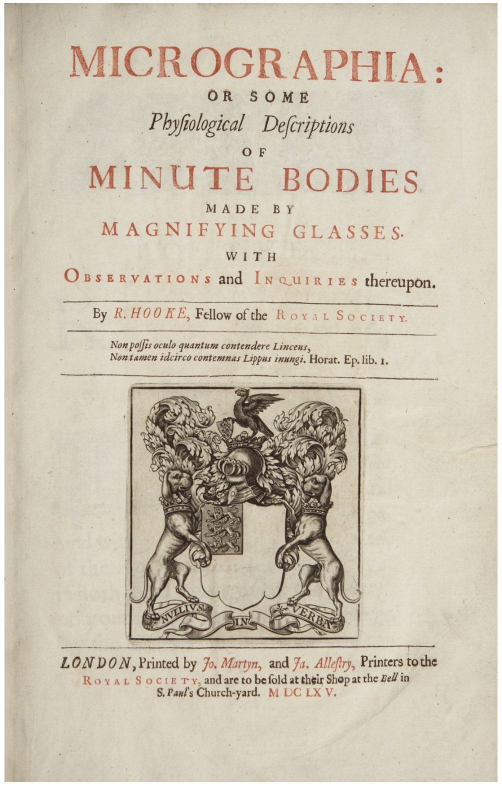
Let's look at the simplest example, a ball lens.



Robert Hooke

- Robert Hooke FRS (18 July 1635 – 3 March 1703) was an English polymath active as a scientist, natural philosopher and architect, who is credited to be one of the first two scientists to discover microorganisms in 1665 using a compound microscope that he built himself.

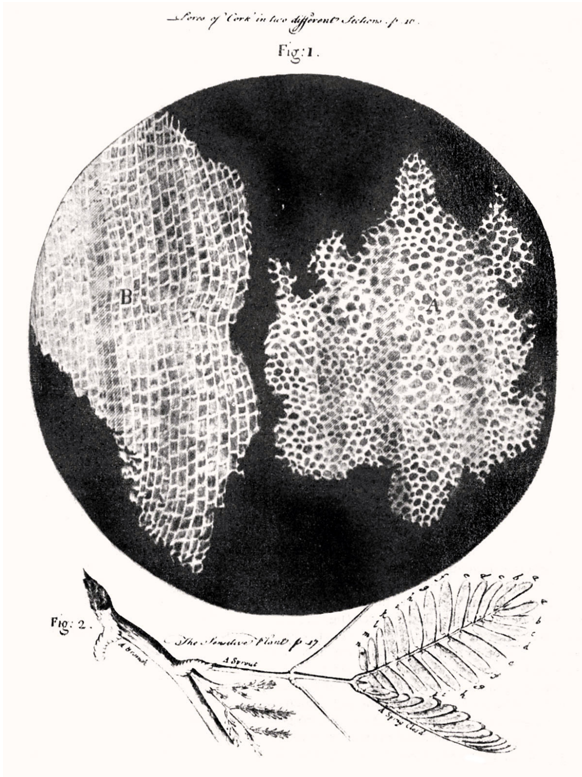
Robert Hooke


MICROGRAPHIA:
OR SOME
Physiological Descriptions
OF
MINUTE BODIES
MADE BY
MAGNIFYING GLASSES.
WITH
OBSERVATIONS and INQUIRIES thereupon.

By *R. HOOKE*, Fellow of the ROYAL SOCIETY.

Non possis oculo quantum contendere Linceus,
Non tamen idcirco contemnas Lippus inungi. Horat. Ep. lib. 1.

LONDON, Printed by *Jo. Martyn*, and *Ja. Allestry*, Printers to the ROYAL SOCIETY, and are to be sold at their Shop at the *Bell* in S. *Paul's* Church-yard. M DC LX V.



Antonie van Leeuwenhoek

- Antonie Philips van Leeuwenhoek (24 October 1632 – 26 August 1723) was a Dutch microbiologist and microscopist.
- A largely self-taught man in science, he is commonly known as "the Father of Microbiology", and one of the first microscopists and microbiologists.
- Using single-lensed microscopes of his own design and make, Van Leeuwenhoek was the first to observe "animalcules", now referred to as unicellular organisms. He was the first to relatively determine their size.

Antonie van Leeuwenhoek

- Perhaps his most famous experiment came in 1674 when he viewed some lake water:

"I now saw very plainly that these were little eels, or worms, lying all huddled up together and wriggling just as if you saw, with thenaked eye, a whole tubful of little eels and water, with the eels squirming among one another; and the whole water seemed to be alive with these multifarious animalcules.

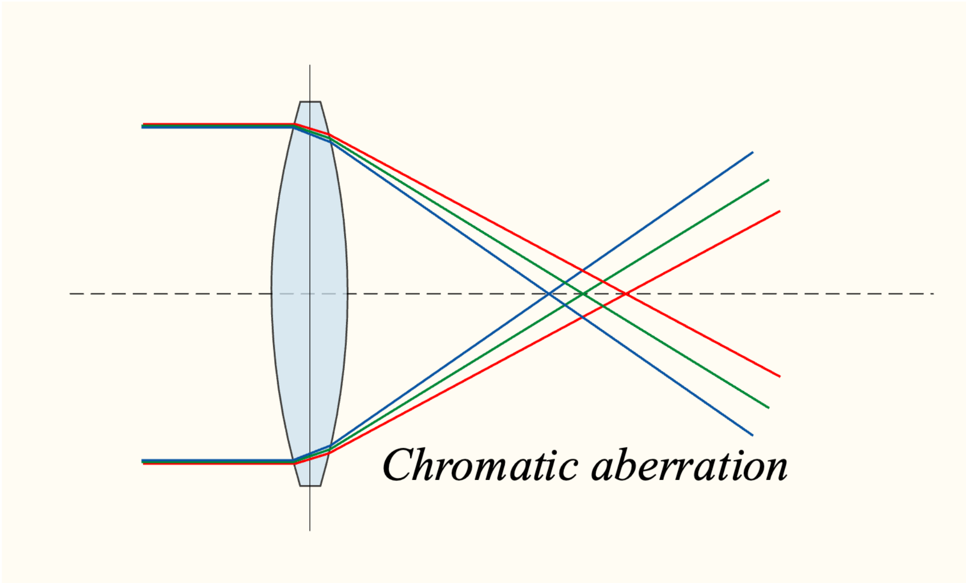
This was for me, among all the marvels that I have discovered in nature, the most marvelous of all; and I must say, for my part, that no more pleasant sight has every yet come before my eyes that these many thousand of living creatures seen all alive in a little drop of water, moving among one another, each several creature having its own proper motion."

First steps in microscopy

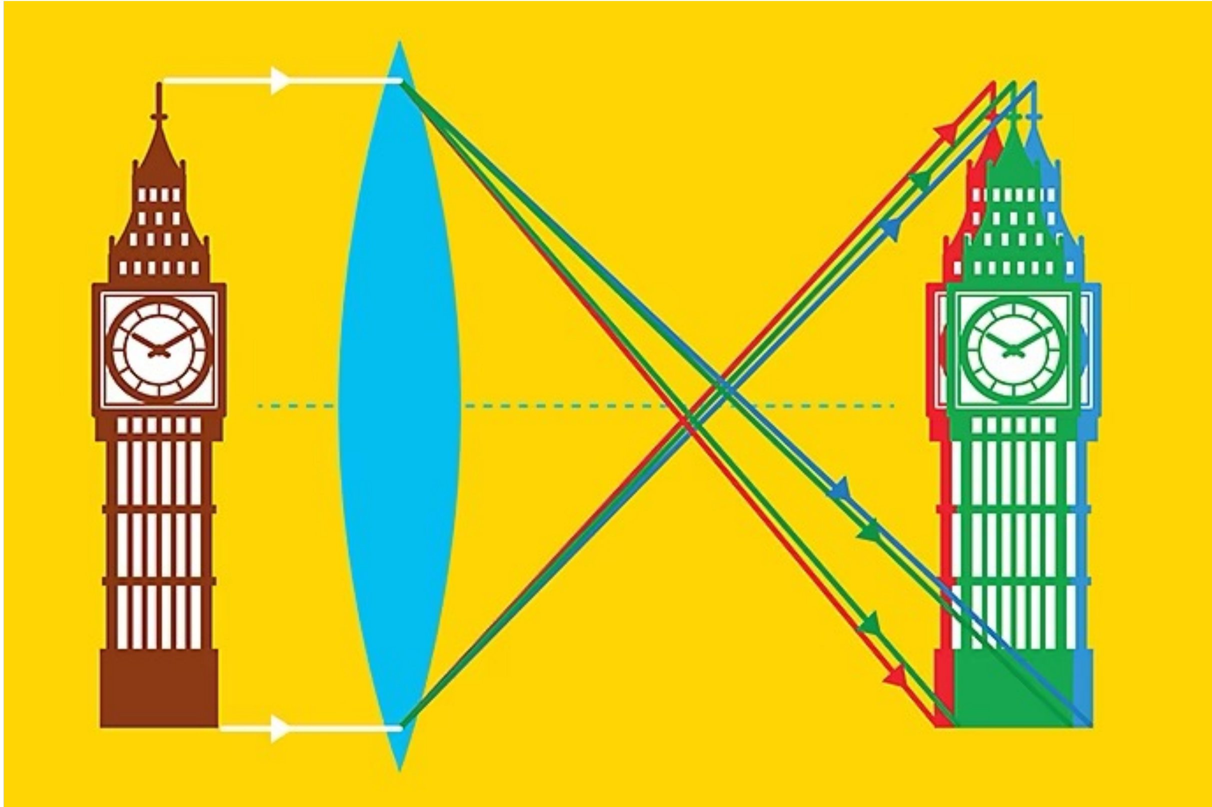
- The next major step in the history of the microscope occurred another 100 years later with the invention of the *achromatic lens* by Chester Moore Hall, in the 1730s.
- Chester Moore Hall (9.12.1703, Leigh, Essex, England – 17.3.1771, Sutton) was a British lawyer and inventor who produced the first achromatic lenses in 1729 or 1733 (accounts differ).
- He discovered that by using a second lens of different shape and refracting properties, he could realign colors with minimal impact on the magnification of the first lens.

First principles

- An achromatic lens or achromat is a lens that is designed to limit the effects of chromatic aberration.
- It happens in presence of non-monochromatic light because the index of refraction of a lens is frequency variable.
- Achromatic lenses are corrected to bring two wavelengths (typically red and blue) into focus on the same plane.
- Wavelengths in between these two then have better focus error than could be obtained with a simple lens.



First principles



Abbe number

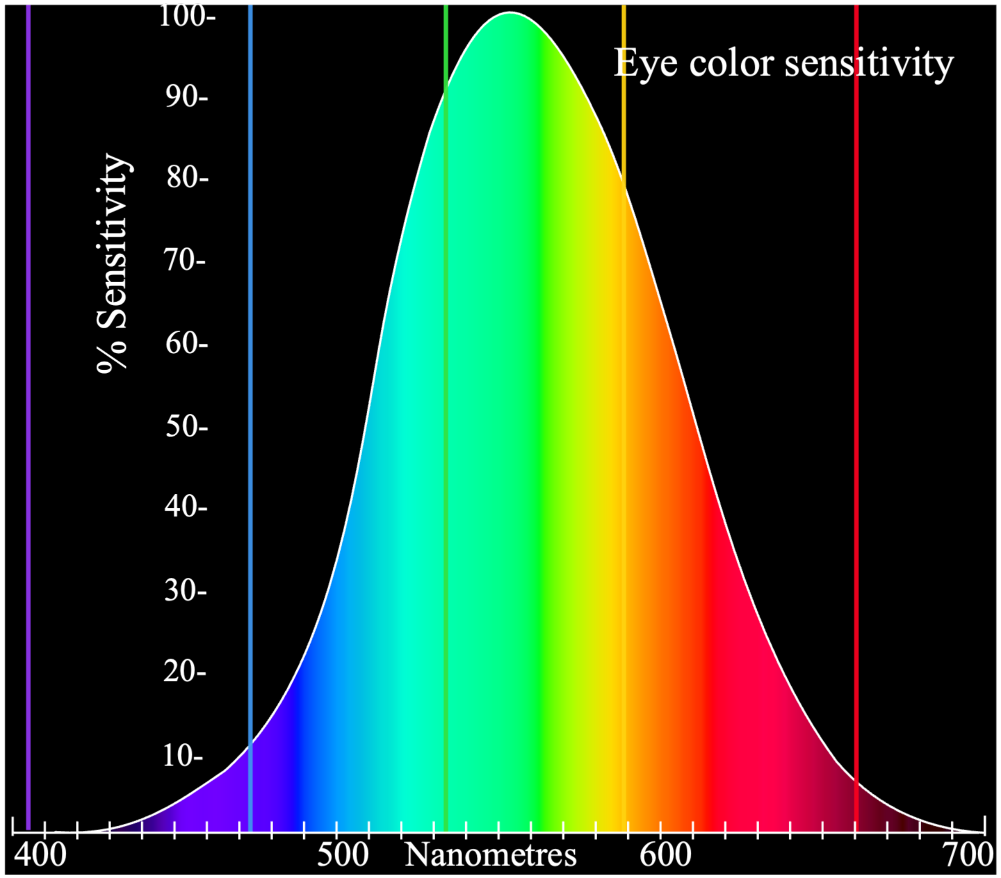
The Abbe number, within the optical region, is defined as follows

$$V_d = \frac{n_d - 1}{n_F - n_C}$$

are the refractive indices of the material at the wavelengths of the Fraunhofer's C, d, and F spectral lines (656.3 nm, 587.56 nm, and 486.1 nm respectively). This formulation only applies to the human vision.

Outside this range requires the use of different spectral lines.

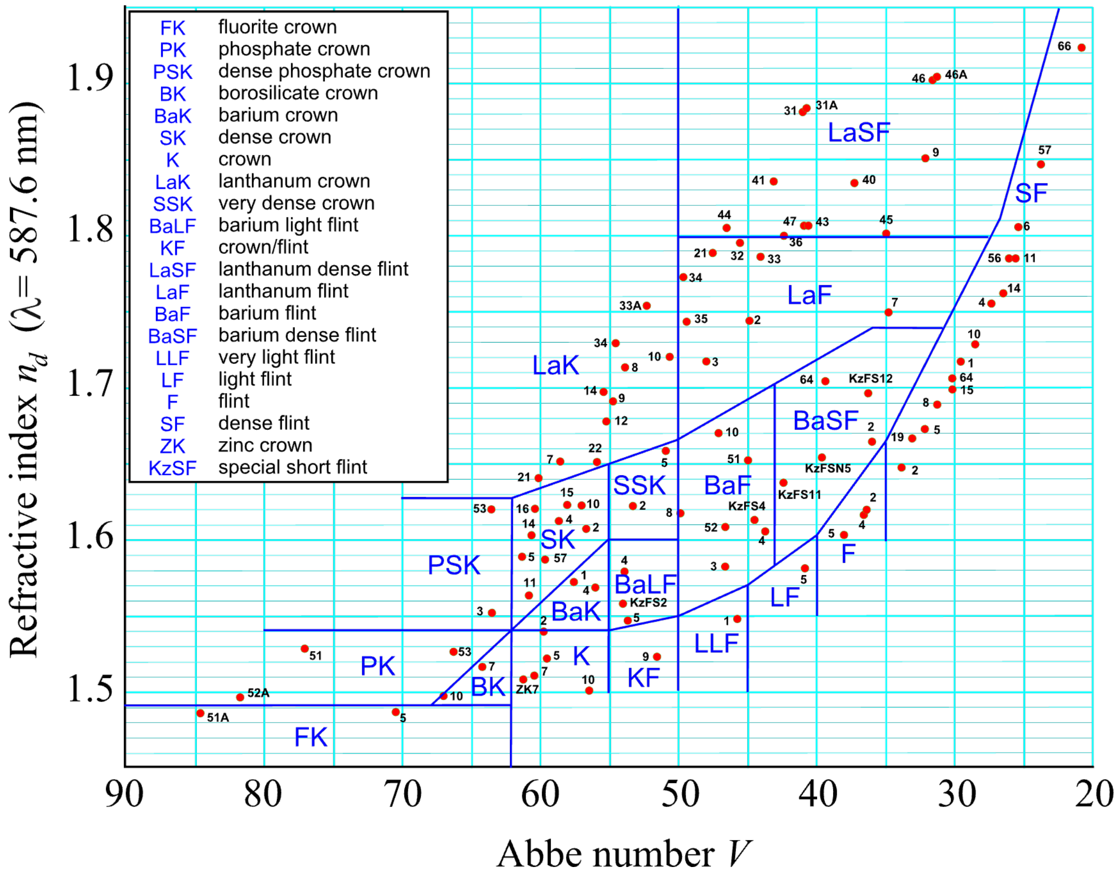
For non-visible spectral lines the term "V-number" is more commonly used.



Abbe diagram

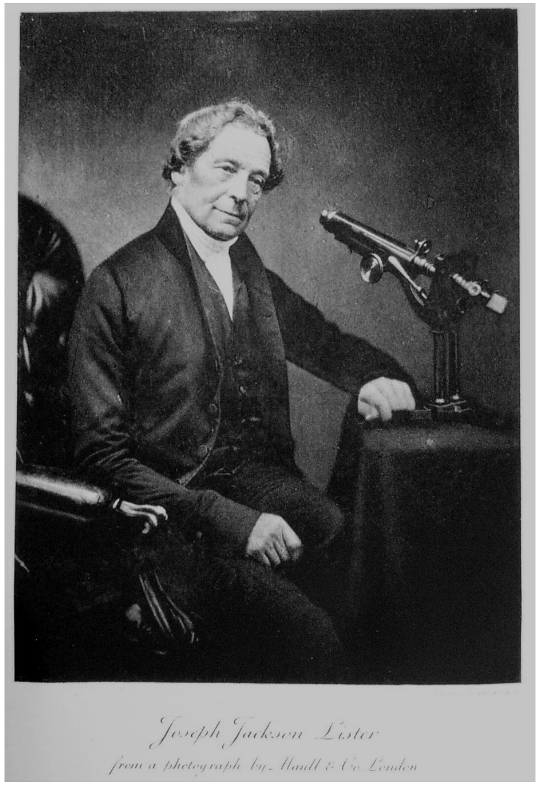
An Abbe diagram plots the *Abbe number V* against refractive index for a range of different glasses (red dots). Glasses are classified using a letter-number code to reflect their composition and position on the diagram. Data plotted from the Schott Glass catalogue.

The Abbe number, or V-number, is a measure of the material's dispersion (change of refractive index versus wavelength), with *high values of V indicating low dispersion*.



First steps in microscopy

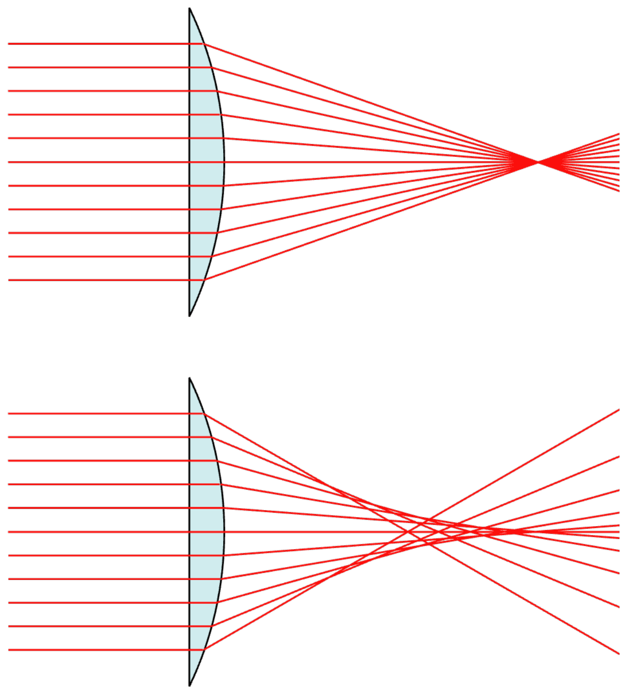
- Joseph Jackson Lister (11.1.1786-24.10.1869), English amateur opticist whose discoveries played an important role in perfecting the objective lens system of the microscope, elevating that instrument to the status of a serious scientific tool.
- in 1830, Joseph Lister solved the problem of *spherical aberration* (light bends at different angles depending on where it hits the lens) by placing lenses at precise distances from each other.



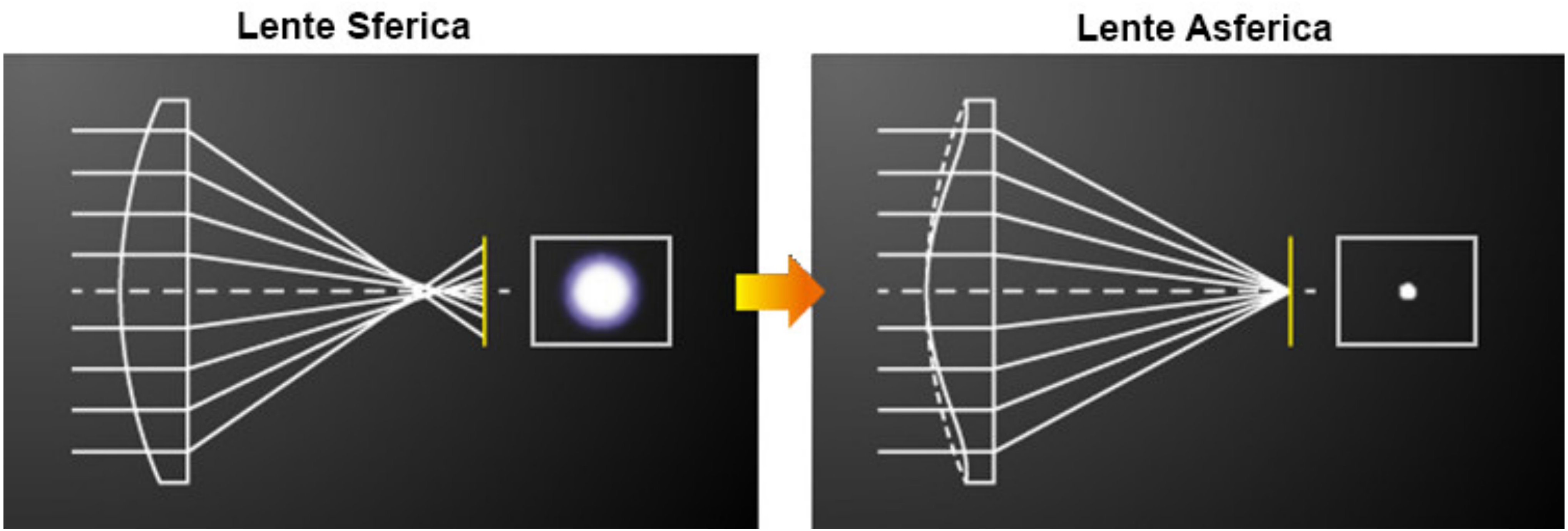
First principles

- *Spherical aberration* is an aberration that is part of *monochromatic aberrations* and axial aberrations. It belongs to optical systems with spherical lenses. These lead to the formation of a distorted image.
- It is caused by the fact that the sphere is not the ideal surface for making a lens, but it is commonly used for construction simplicity.
- The rays far from the axis are focused at different distances with respect with the central ones.

Ideal vs spherically aberrated lens



First principles



First principles

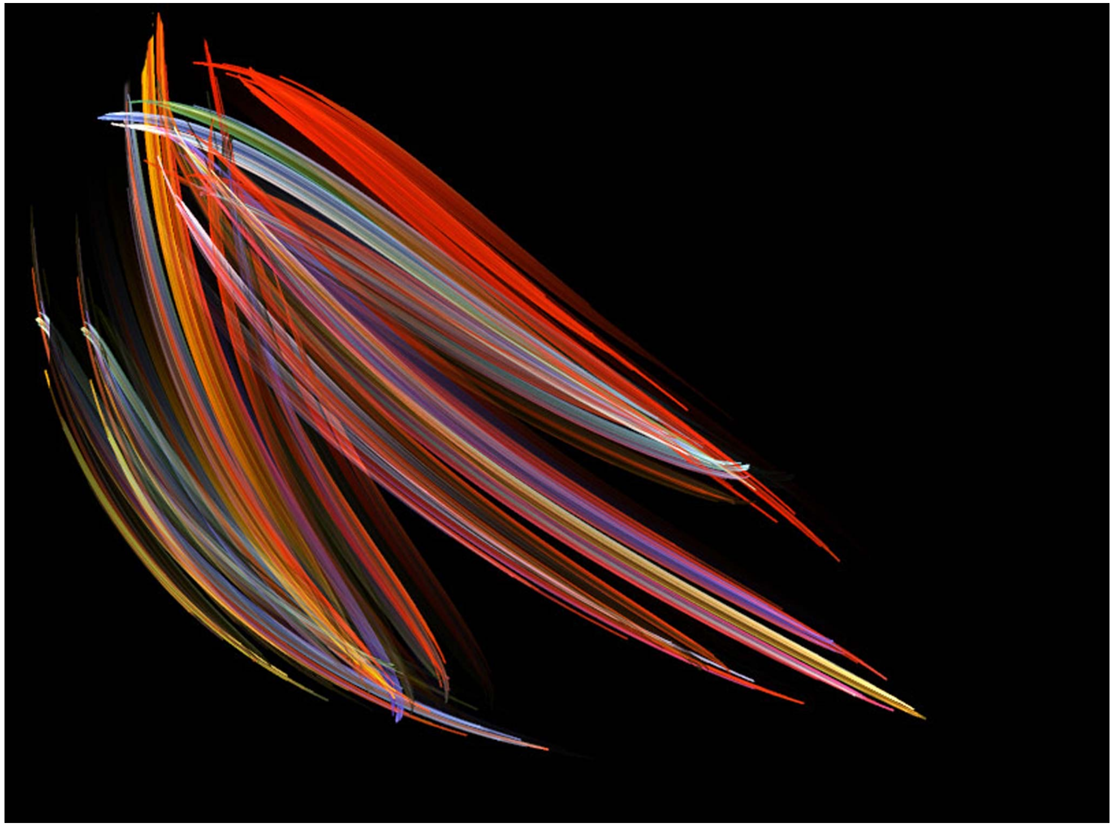
Such technical advancements contributed towards a marked improvement in the quality of images.

Previously, due to the poor quality of glass and imperfect lens, microscopists had been viewing nothing but distorted images - somewhat like the first radios were extremely crackly.

1st Place 2008 Photomicrography Competition

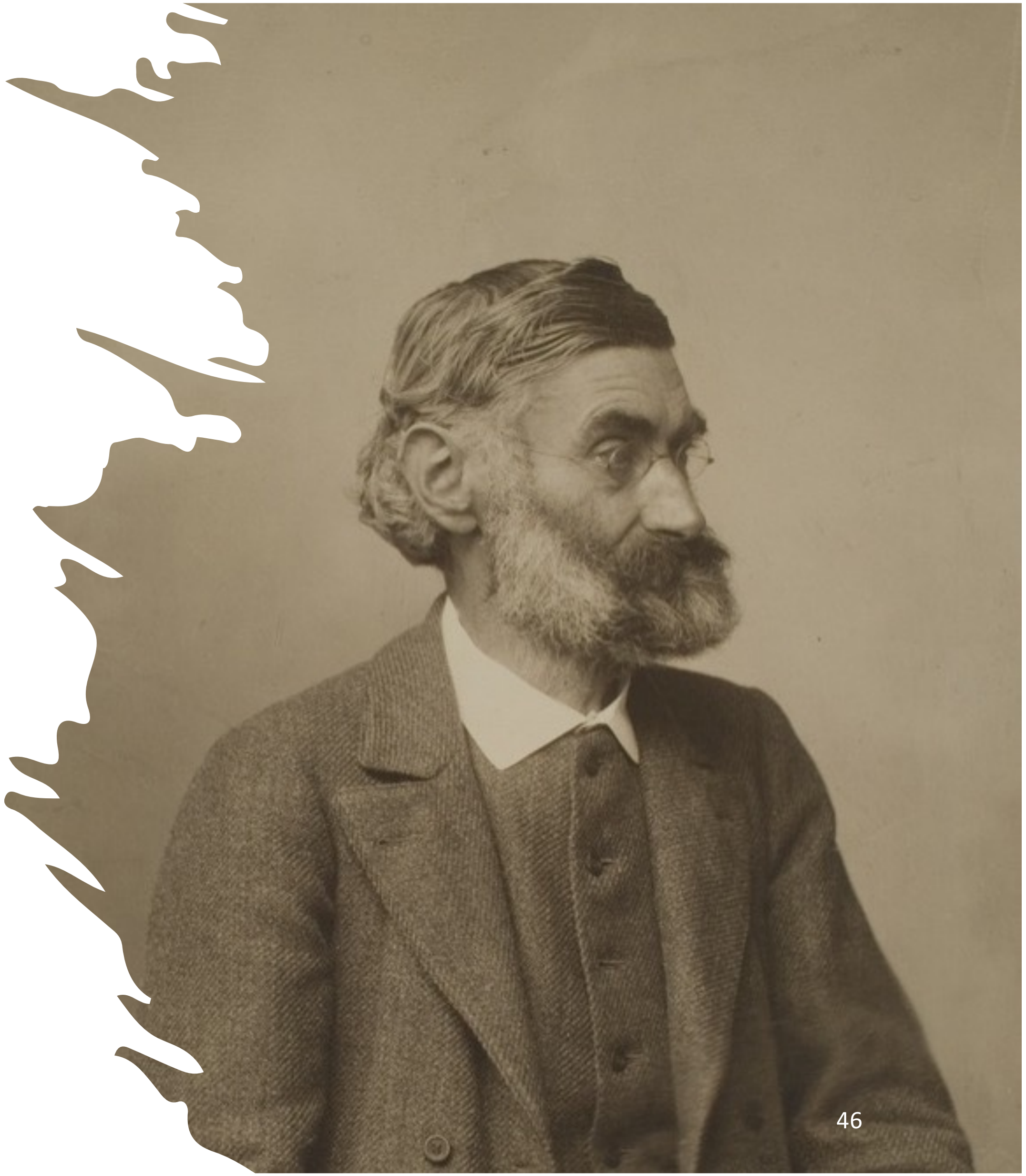
Pleurosigma (marine diatoms) by Michael J. Stringer in UK

Technique: Darkfield, Polarized Light, Magnification: 200x



Ernst Abbe

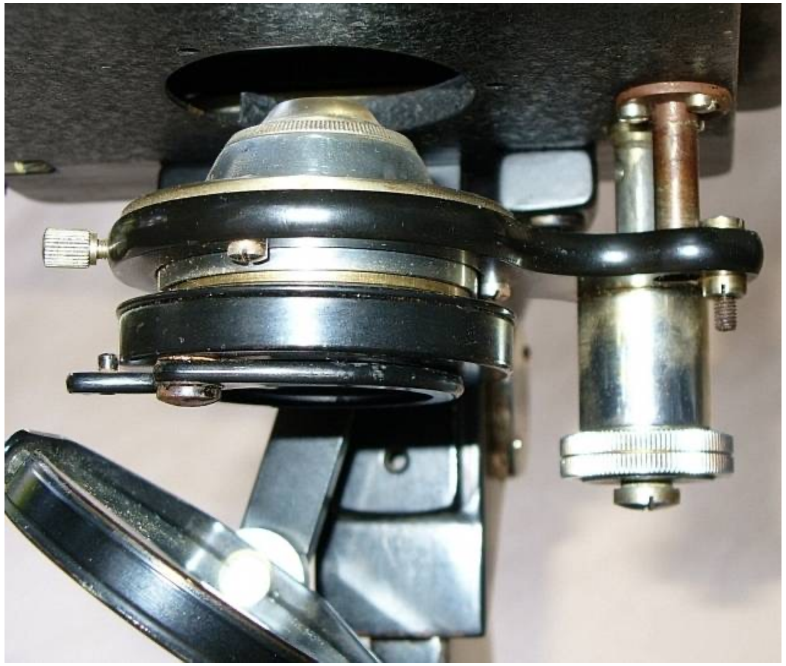
- Ernst Karl Abbe (23 January 1840 – 14 January 1905) was a German physicist, optical scientist, entrepreneur.
- Together with Otto Schott and Carl Zeiss, he developed numerous optical instruments.
- He was also a co-owner of Carl Zeiss AG, a German manufacturer of scientific microscopes, astronomical telescopes, planetariums, and other advanced optical systems.



Ernst Abbe

- Ernst Abbe made clear the difference between magnification and resolution and criticized the practice of using eyepieces with too high a magnification as "empty magnification."
- By 1869, his work produced a new patented illumination device - the Abbe condenser.

A condenser between the stage and mirror of a vintage microscope



Microscope condenser

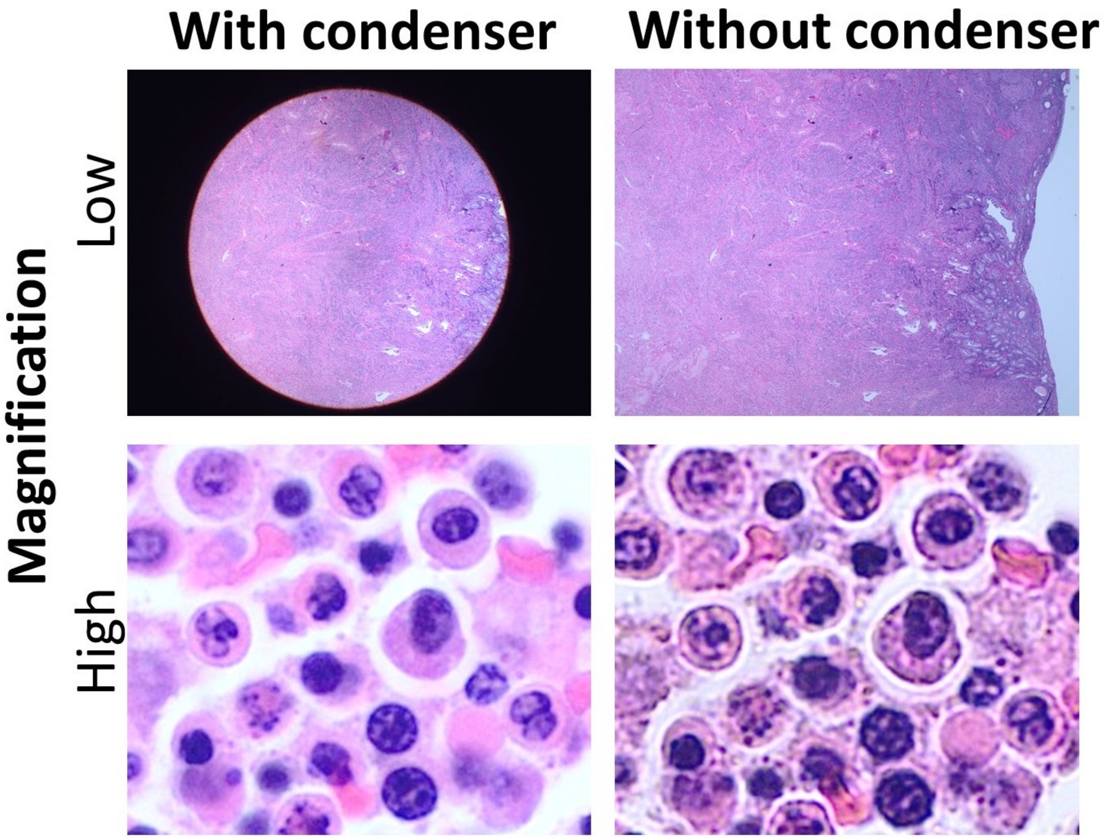
- Condensers are located above the light source and under the sample in an upright microscope, and above the stage and below the light source in an inverted microscope.
- They act to gather light from the microscope's light source and concentrate it into a cone of light that illuminates the specimen.
- The aperture and angle of the light cone must be adjusted (via the size of the diaphragm) for each different objective lens with different numerical apertures.

Microscope condenser

Light microscopy with and without condenser.

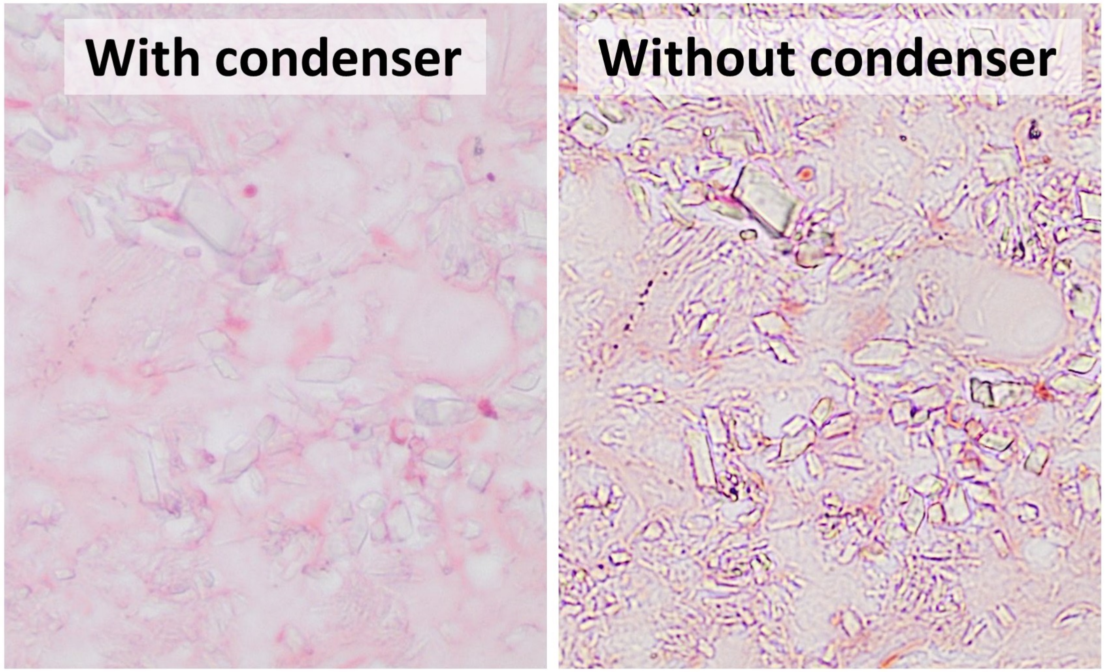
At low magnification, using a condenser may limit the field of view, and in such cases it is preferable to not use it.

At high magnification, a condenser makes borders less marked, and is generally preferable in such cases.



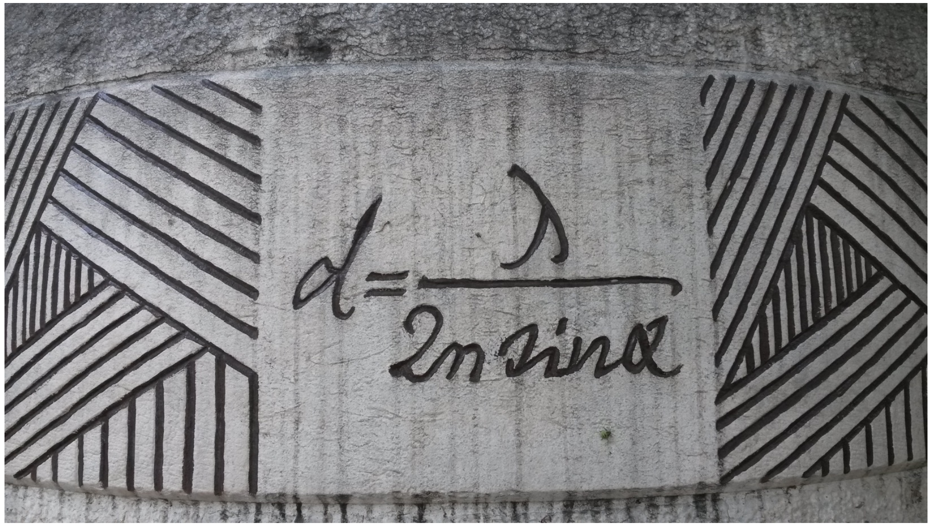
Microscope condenser

An example of a situation where microscopy without condenser is preferable at high magnification is the evaluation of crystals (calcium pyrophosphate dihydrate crystal deposition disease pictured).



Ernst Abbe

The resolution limit formula engraved in an Ernst Abbe memorial in Jena, Germany



Microscope by Carl Zeiss (1879) with optics by Abbe

Ernst Abbe

- The capacity of an optical microscope to resolve fine details depends on the color of the light (λ), by the media the light propagates (*n*), and by the angle of light traveling through the lens with respect to the normal.

$$d = \frac{\lambda}{2nsin\alpha}$$

- The term n sinα is also known as numerical aperture (NA).

Ernst Abbe memorial stone at the Friedrich Schiller University of Jena, built 1977

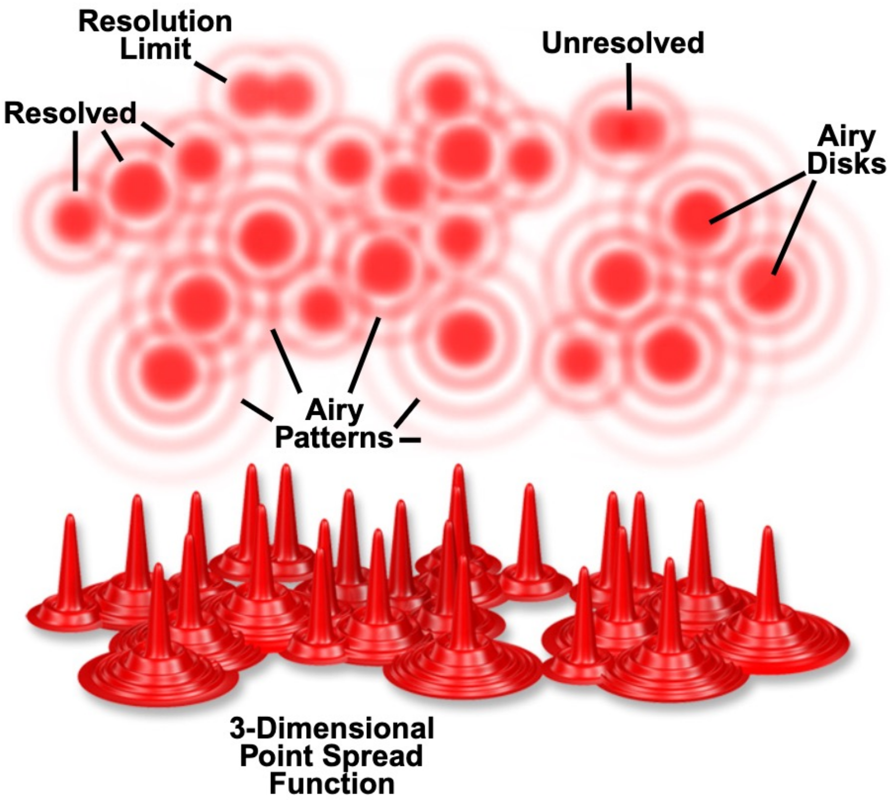
Resolution

Airy Patterns and the Limit of Resolution

The limit of resolution of a microscope objective refers to its ability to distinguish between two closely spaced Airy disks in the diffraction pattern (noted in the figure).

Three-dimensional representations of the diffraction pattern near the intermediate image plane are known as the point spread function, and are illustrated in the lower portion of Figure.

The specimen image is represented by a series of closely spaced point light sources that form Airy patterns and is illustrated in both two and three dimensions.

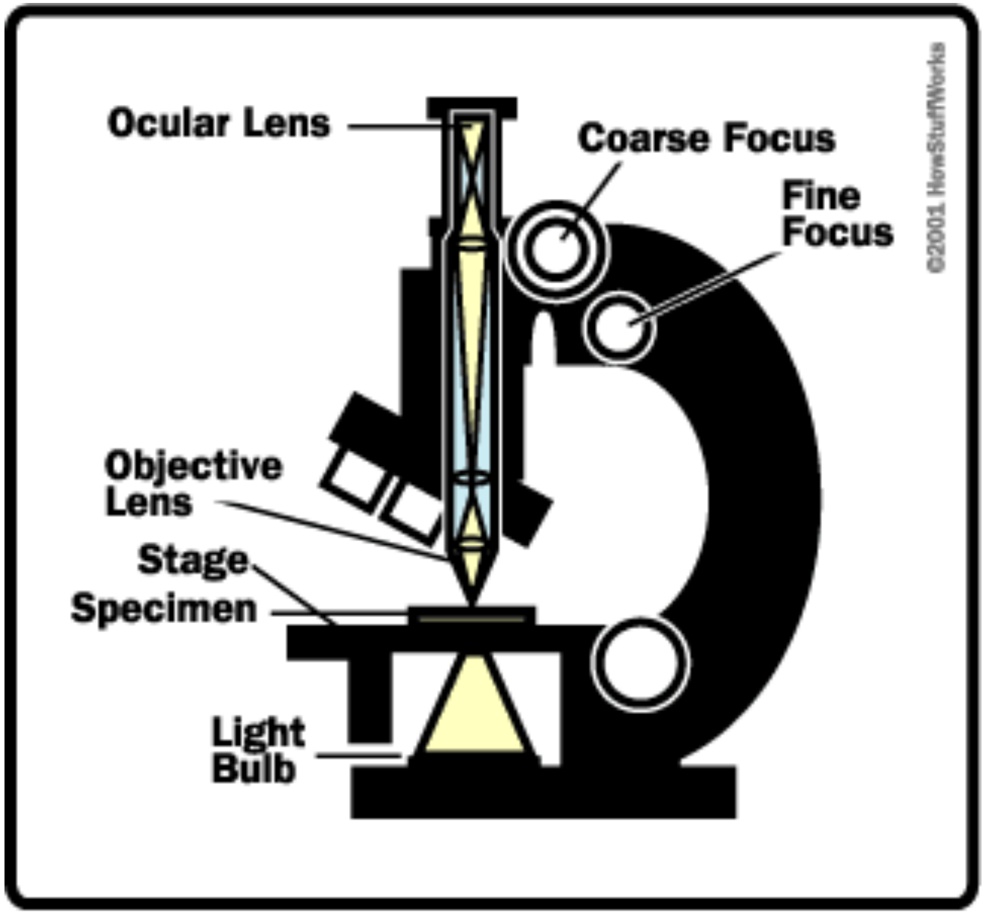


Key points

- Magnification is the ability to make small objects seem larger, such as making a microscopic organism visible.
- Resolution is the ability to distinguish two objects from each other.
- Light microscopy has limits to both its resolution and its magnification.

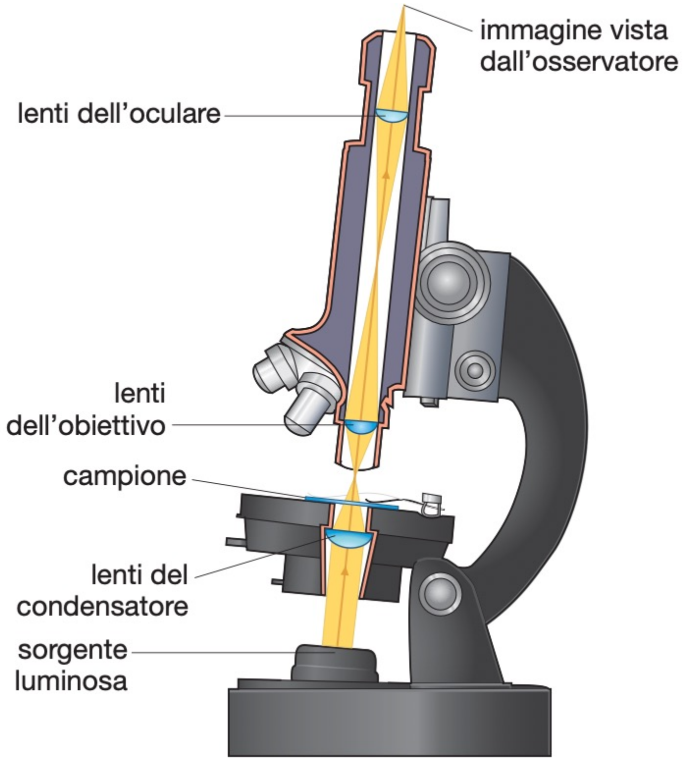
Optical microscopes

- Optical microscopes are the oldest design of microscope and were possibly invented in their present compound form in the 17th century.
- Basic optical microscopes can be very simple, although many complex designs aim to improve resolution and sample contrast.
- The object is placed on a stage and may be directly viewed through one or two ocular lenses on the microscope



Optical microscopes

- Most cells are so small that they cannot be seen with the naked eye. To see a cell clearly, an instrument that magnifies the image is used: the microscope.



Optical microscopes

- The sample can be lit in a variety of ways. Transparent objects can be lit from below and solid objects can be lit with light coming through (bright field) or around (dark field) the objective lens.
- A range of objective lenses with different magnification are usually provided mounted on a turret, allowing them to be rotated into place and providing an ability to zoom-in. The maximum magnification power of optical microscopes is typically limited to around 1000x.

Conclusions