Lecture 1
The Principles of Microscopy

- **BMS 524 - "Introduction to Confocal Microscopy and Image Analysis"**

Purdue University Department of Basic Medical Sciences,
School of Veterinary Medicine

J.Paul Robinson, Ph.D.

- Professor of Immunopharmacology
- Director, Purdue University Cytometry Laboratories

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Evaluation

- End of term quiz - 100% grade

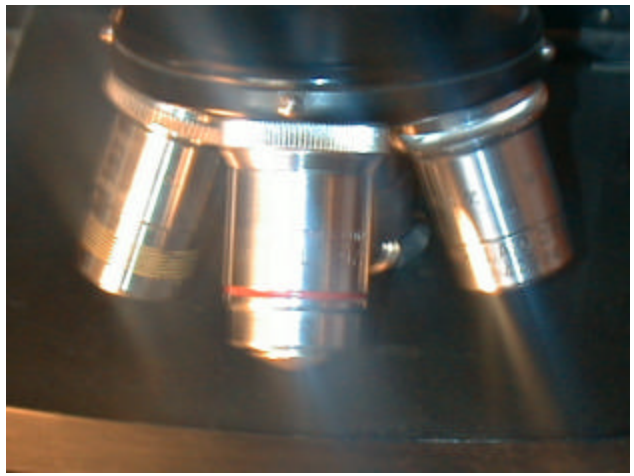
Introduction to the Course

- Microscopy
- Fluorescence
- Basic Optics
- Confocal Microscopes
- Basic Image Analysis
- 3D image analysis
- Live Cell Studies
- Advanced Applications

Introduction to Lecture 1

- Early Microscopes
- Modern Microscopes
- Magnification
- Nature of Light
- Optical Designs

Microscopes



- Upright
- Inverted
- Köhler Illumination
- Fluorescence Illumination

"Microscope" was first coined by members of the first "*Academia dei Lincei*" a scientific society which included Galileo

Earliest Microscopes

- 1590 - Hans & Zacharias Janssen of Middleburg, Holland manufactured the first compound microscopes
- **1660** - Marcello Malpighi **circa 1660,** was one of the first great microscopists, considered the father embryology and early histology - observed capillaries in 1660
- 1665 - Robert Hooke (1635-1703)- book *Micrographia*, published in 1665, devised the compound microscope most famous microscopical observation was his study of thin slices of cork. He wrote:

". . . I could exceedingly plainly perceive it to be all perforated and porous. . . these pores, or cells, . . . were indeed the first microscopical pores I ever saw, and perhaps, that were ever seen, for I had not met with any Writer or Person, that had made any mention of them before this."

Earliest Microscopes

•1673 - Antioni van Leeuwenhoek (1632-1723) Delft, Holland, worked as a draper (a fabric merchant); he is also known to have worked as a surveyor, a wine assayer, and as a minor city official.

•Leeuwenhoek is incorrectly called "*the inventor of the microscope*"

•Created a “simple” microscope that could magnify to about 275x, and published drawings of microorganisms in 1683

•Could reach magnifications of over 200x with simple ground lenses - however compound microscopes were mostly of poor quality and could only magnify up to 20-30 times. Hooke claimed they were too difficult to use - his eyesight was poor.

•Discovered bacteria, free-living and parasitic microscopic protists, sperm cells, blood cells, microscopic nematodes

•In 1673, Leeuwenhoek began writing letters to the Royal Society of London - published in *Philosophical Transactions of the Royal Society*

•In 1680 he was elected a full member of the Royal Society, joining Robert Hooke, Henry Oldenburg, Robert Boyle, Christopher Wren

Secondary Microscopes

- **George Adams Sr.** made many microscopes from about 1740-1772 but he was predominantly just a good manufacturer not inventor (in fact it is thought he was more than a copier!)
- Simple microscopes could attain around 2 micron resolution, while the best compound microscopes were limited to around 5 microns because of chromatic aberration
- In the 1730s a barrister names **Chester More Hall** observed that flint glass (newly made glass) dispersed colors much more than "crown glass" (older glass). He designed a system that used a concave lens next to a convex lens which could realign all the colors. This was the first ***achromatic lens***. **George Bass** was the lens-maker that actually made the lenses, but he did not divulge the secret until over 20 years later to **John Dolland** who copied the idea in 1759 and patented the achromatic lens.
- In 1827 **Giovanni Battista Amici**, built high quality microscopes and introduced the first matched ***achromatic microscope*** in 1827. He had previously (1813 designed "*reflecting microscopes*" using curved mirrors rather than lenses. He recognized the importance of coverslip thickness and developed the concept of "**water immersion**"

Lister, Abbe, Zeiss & Schott

- In 1830, by **Joseph Jackson Lister** (father of Lord Joseph Lister) solved the problem of ***Spherical Aberration*** - caused by light passing through different parts of the same lens. He solved it mathematically and published this in the *Philosophical Transactions* in 1830
- **Ernst Abbe** together with **Carl Zeiss** published a paper in 1877 defining the physical laws that determined resolving distance of an objective. Known as Abbe's Law

  "minimum resolving distance *(d)* is related to the wavelength of light *(lambda)* divided by the $d = \frac{\lambda}{2 n \sin\theta}$ Numeric Aperture, which is proportional to the angle of the light cone *(theta)* formed by a point on the object, to the objective".
- **Abbe and Zeiss** developed oil immersion systems by making oils that matched the refractive index of glass. Thus they were able to make the a Numeric Aperture (N.A.) to the maximum of 1.4 allowing light microscopes to resolve two points distanced only 0.2 microns apart (the theoretical maximum resolution of visible light microscopes). **Leitz** was also making microscope at this time.
- Dr Otto Schott formulated glass lenses that color-corrected objectives and produced the first "apochromatic" objectives in 1886.

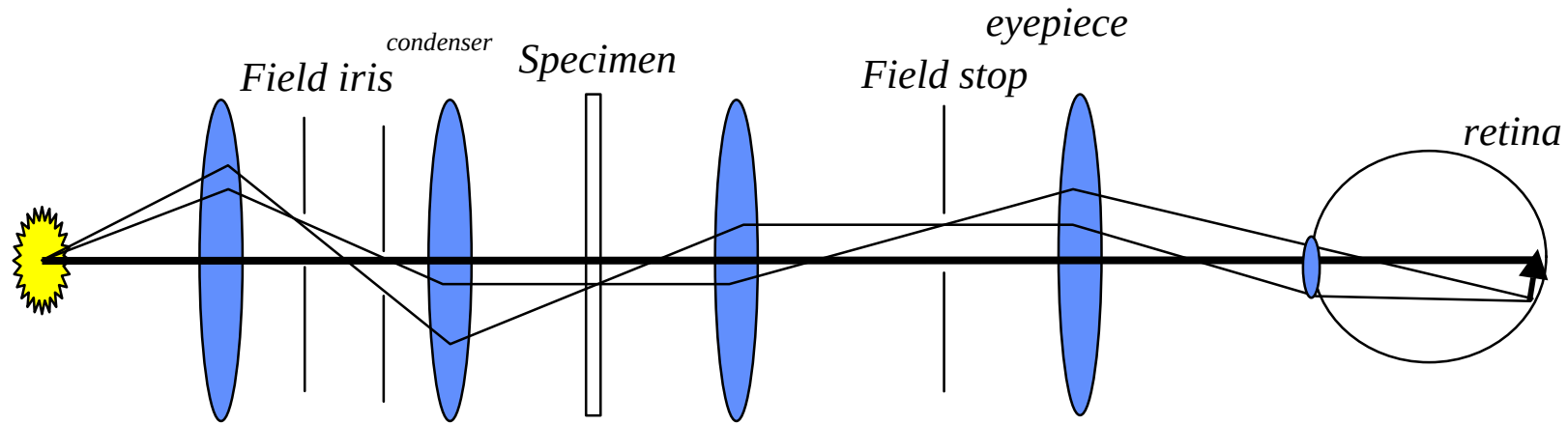
Modern Microscopes

- Early 20th Century Professor Köhler developed the method of illumination still called "Köhler Illumination"
- Köhler recognized that using shorter wavelength light (UV) could improve resolution

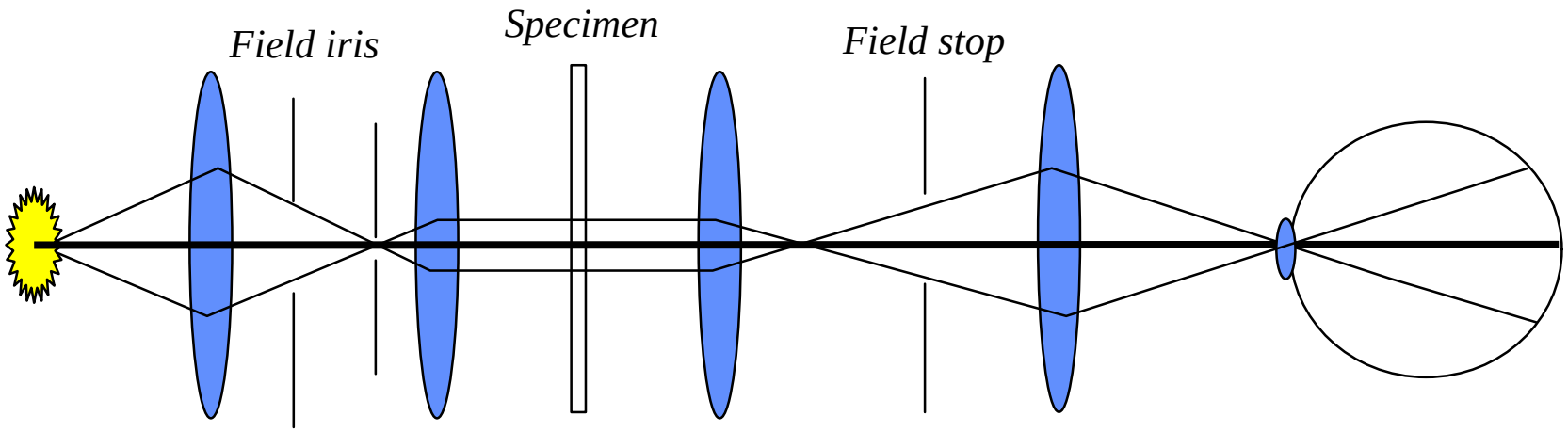
Köhler

- Köhler illumination creates an evenly illuminated field of view while illuminating the specimen with a very wide cone of light
- Two conjugate image planes are formed
  - one contains an image of the specimen and the other the filament from the light

Köhler Illumination



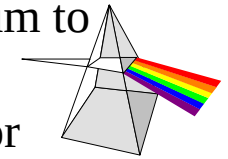
Conjugate planes for image-forming rays



Conjugate planes for illuminating rays

Some Definitions

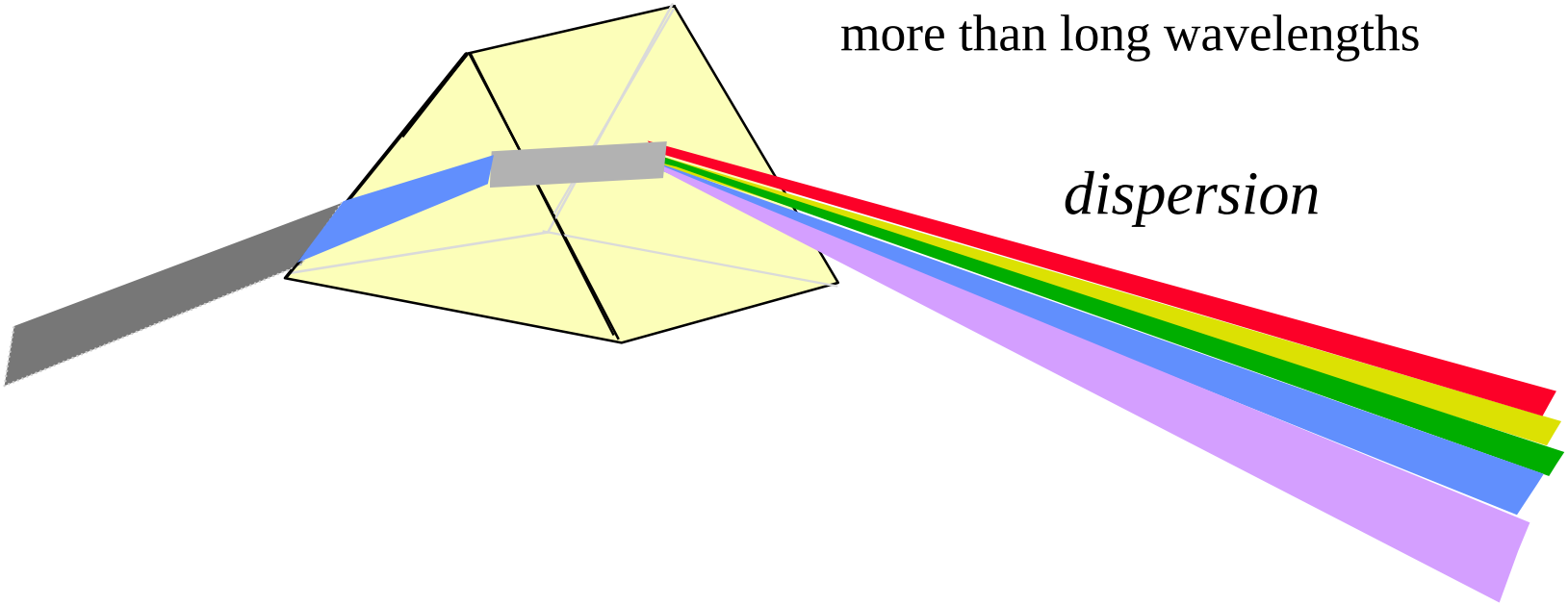
- **Absorption**
  - When light passes through an object the intensity is reduced depending upon the color absorbed. Thus the selective absorption of white light produces colored light.
- **Refraction**
  - Direction change of a ray of light passing from one transparent medium to another with different optical density. A ray from less to more dense medium is bent perpendicular to the surface, with greater deviation for shorter wavelengths



- **Diffraction**
  - Light rays bend around edges - new wavefronts are generated at sharp edges - the smaller the aperture the lower the definition
- **Dispersion**
  - Separation of light into its constituent wavelengths when entering a transparent medium - the change of refractive index with wavelength, such as the spectrum produced by a prism or a rainbow

Refraction

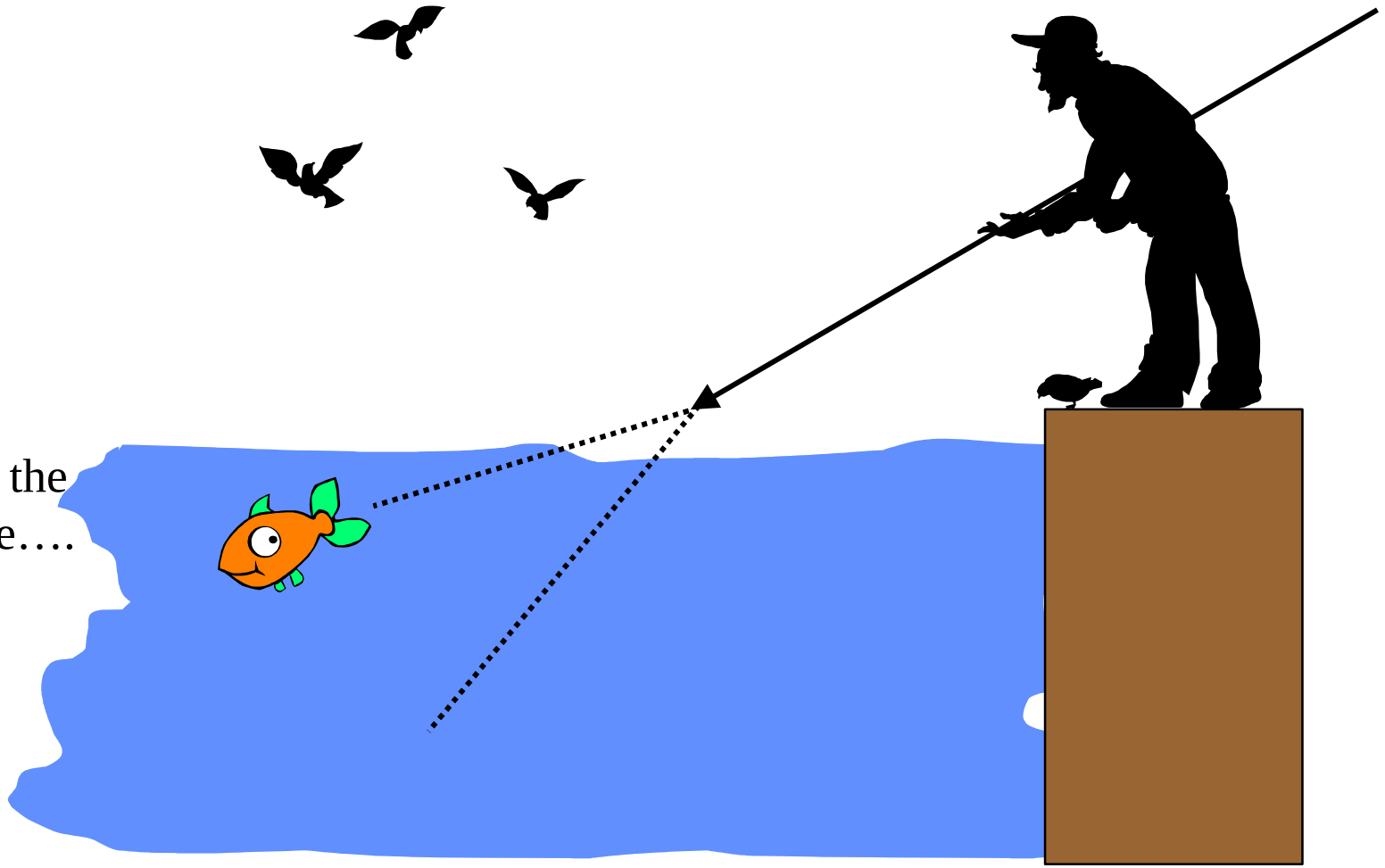
Short wavelengths are “bent” more than long wavelengths



dispersion

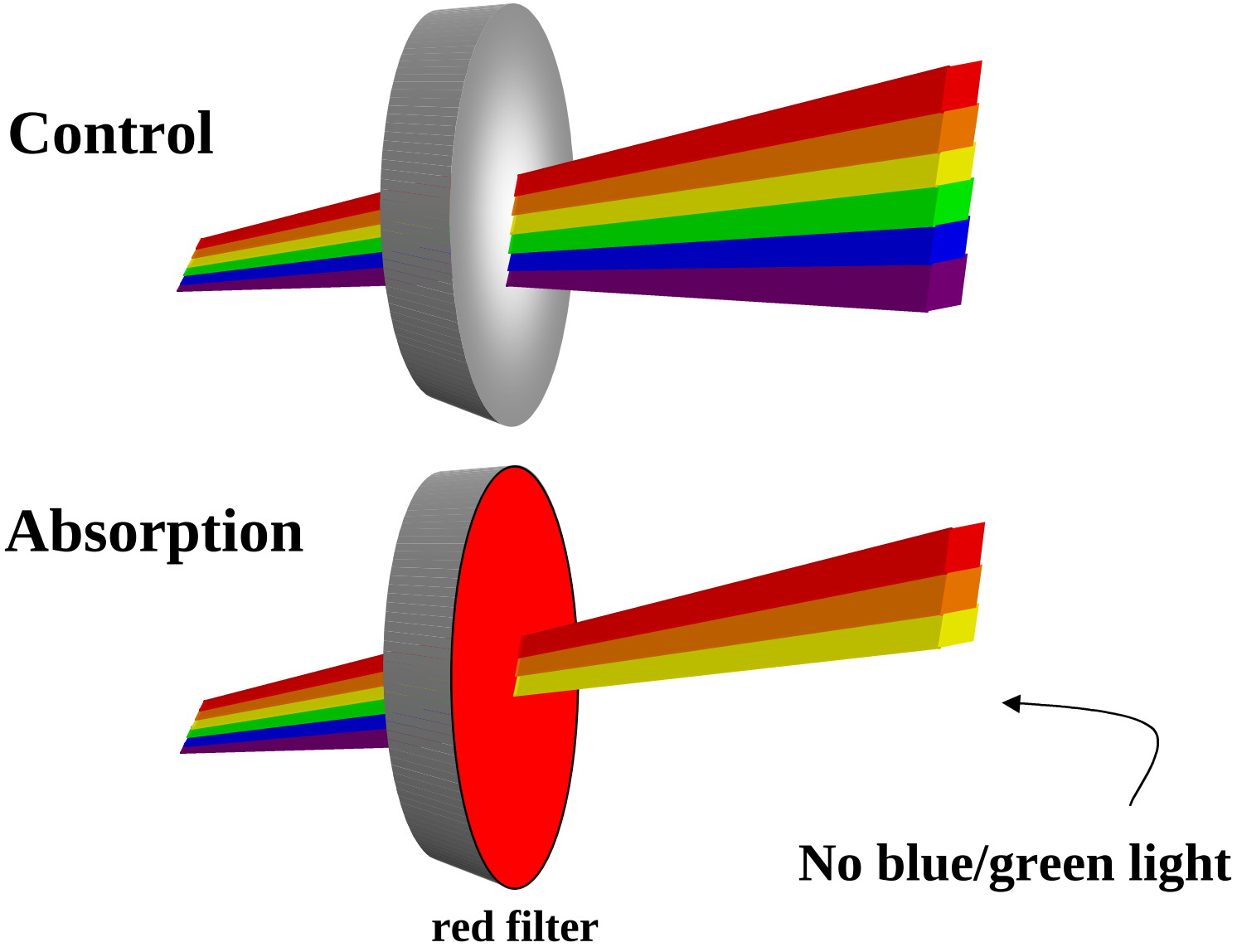
Light is “bent” and the resultant colors separate (dispersion). Red is least refracted, violet most refracted.

Refraction

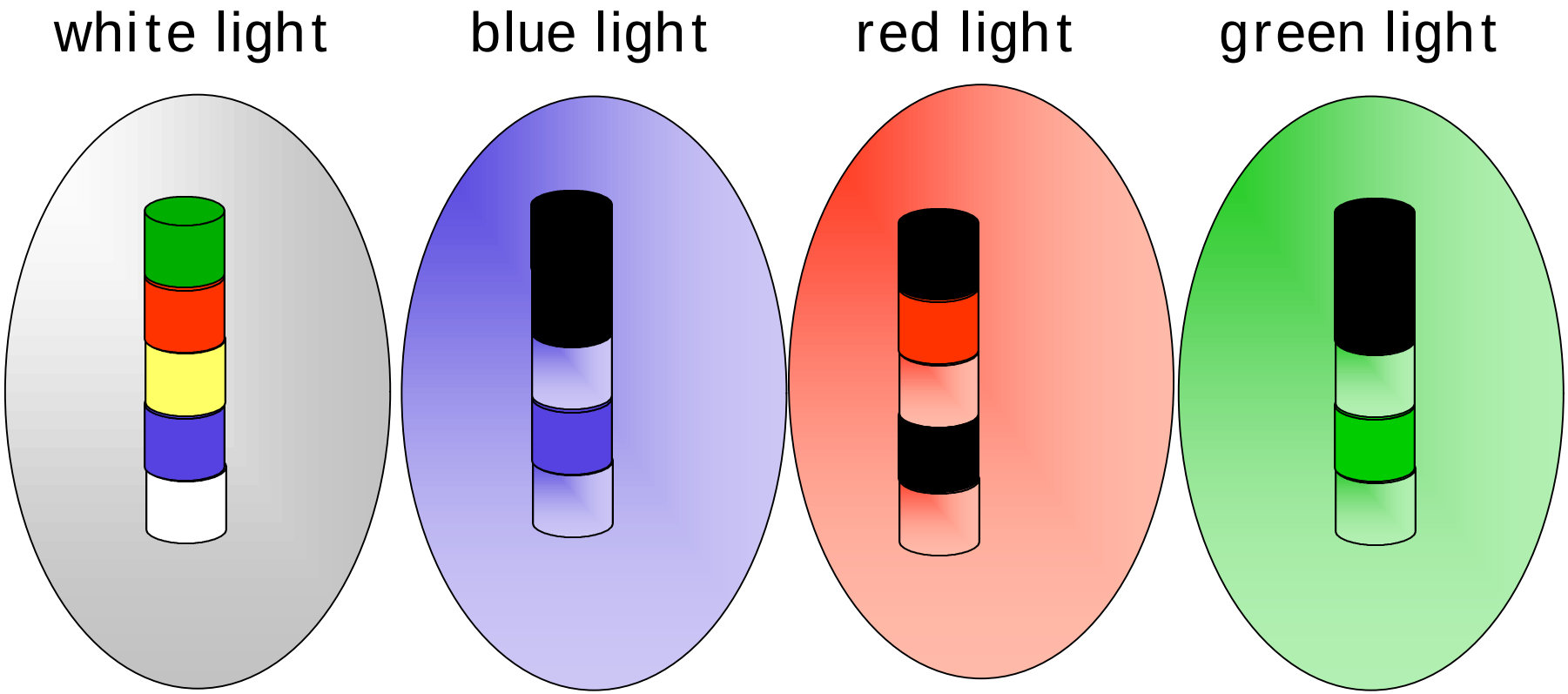


He sees the fish here….

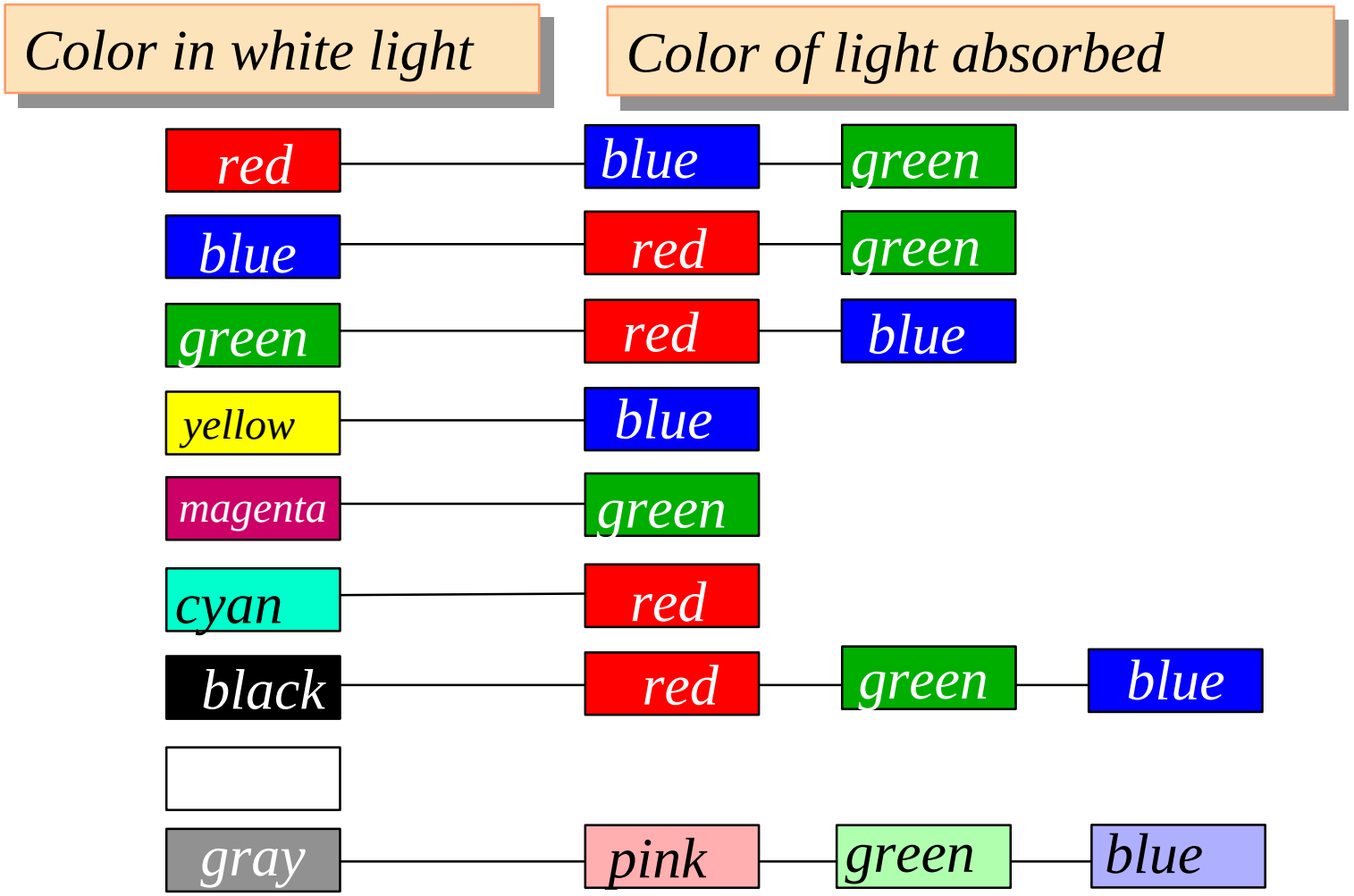
But it is really here!!


Control
Absorption
No blue/green light
red filter

Light absorption



Absorption Chart



The light spectrum

Wavelength ---- Frequency

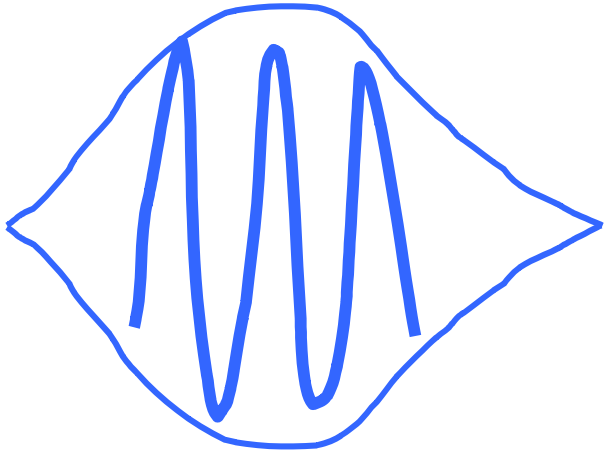
Blue light

488 nm

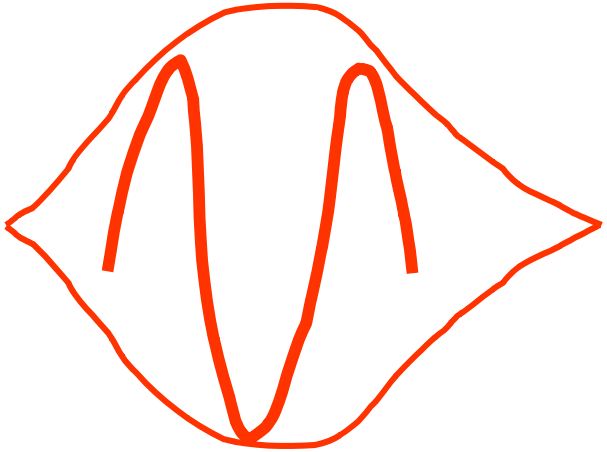
short wavelength

high frequency

high energy (2 times the red)



Photon as a wave packet of energy



Red light

650 nm

long wavelength

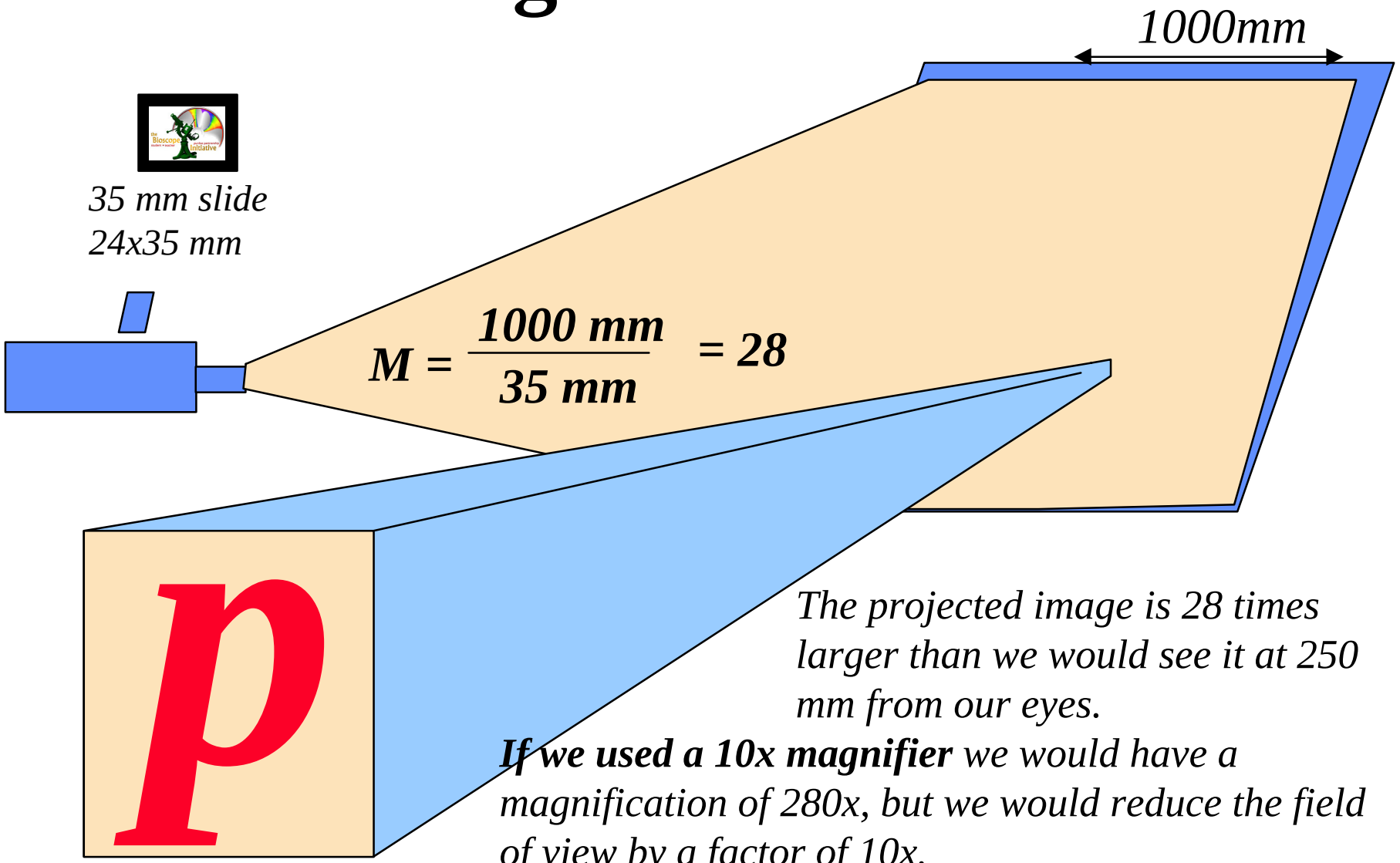
low frequency

low energy

Magnification

- An object can be focussed generally no closer than 250 mm from the eye (depending upon how old you are!)
- this is considered to be the normal viewing distance for 1x magnification
- Young people may be able to focus as close as 125 mm so they can magnify as much as 2x because the image covers a larger part of the retina - that is it is "magnified" at the place where the image is formed

Magnification



The projected image is 28 times larger than we would see it at 250 mm from our eyes.

If we used a 10x magnifier *we would have a magnification of 280x, but we would reduce the field of view by a factor of 10x.*

Some Principles

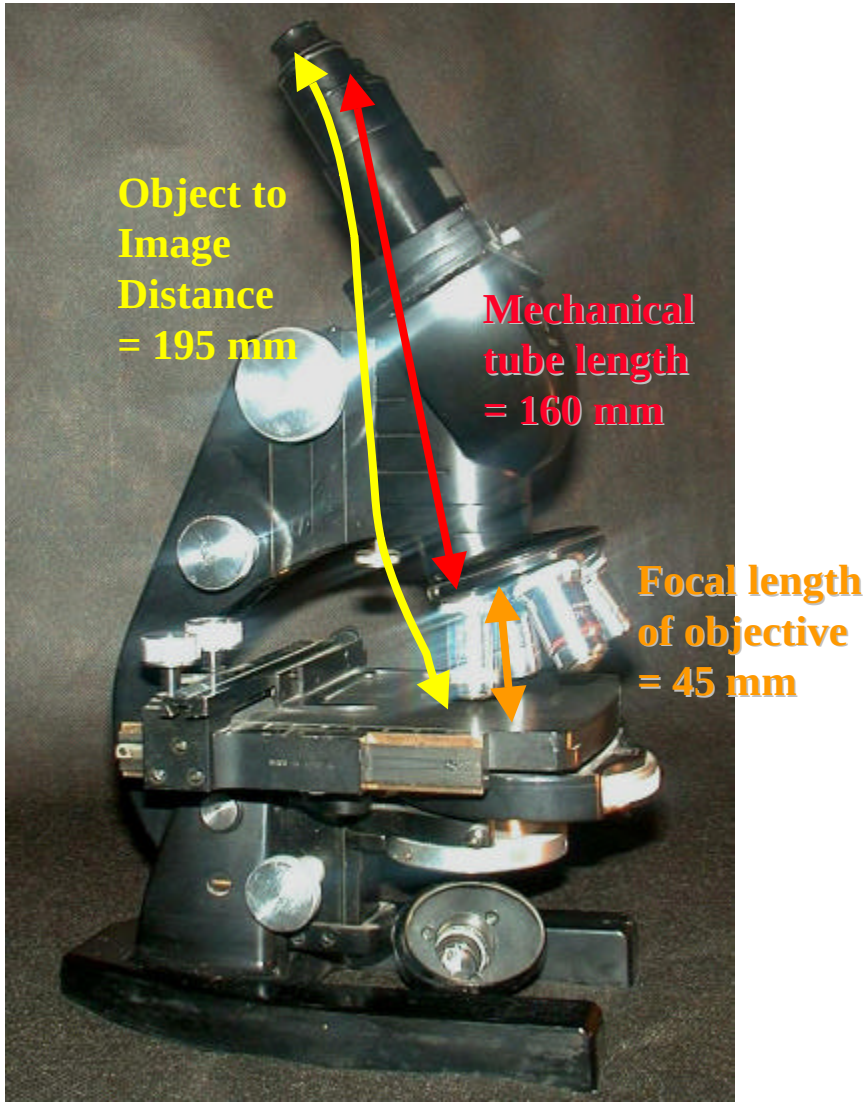
- **Rule of thumb** is is not to exceed 1,000 times the NA of the objective
- Modern microscopes magnify both in the objective and the ocular and thus are called "compound microscopes" - Simple microscopes have only a single lens

Basic Microscopy

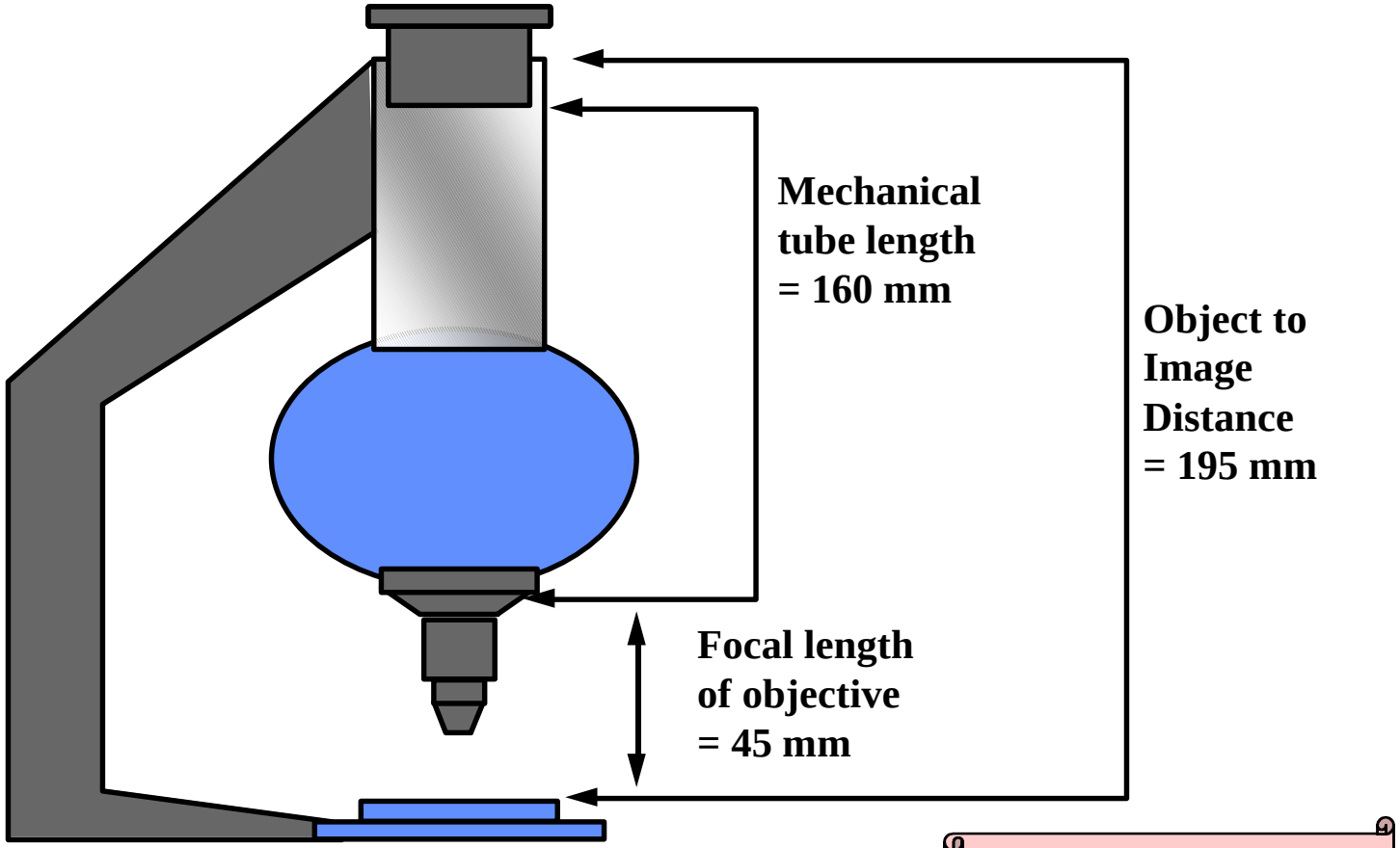
- Bright field illumination does not reveal differences in brightness between structural details - i.e. no contrast
- Structural details emerge via phase differences and by staining of components
- The edge effects (diffraction, refraction, reflection) produce contrast and detail

Microscope Basics

- Originally conformed to the German DIN standard
- Standard required the following
  - real image formed at a tube length of 160mm
  - the parfocal distance set to 45 mm
  - object to image distance set to 195 mm
- Currently we use the ISO standard

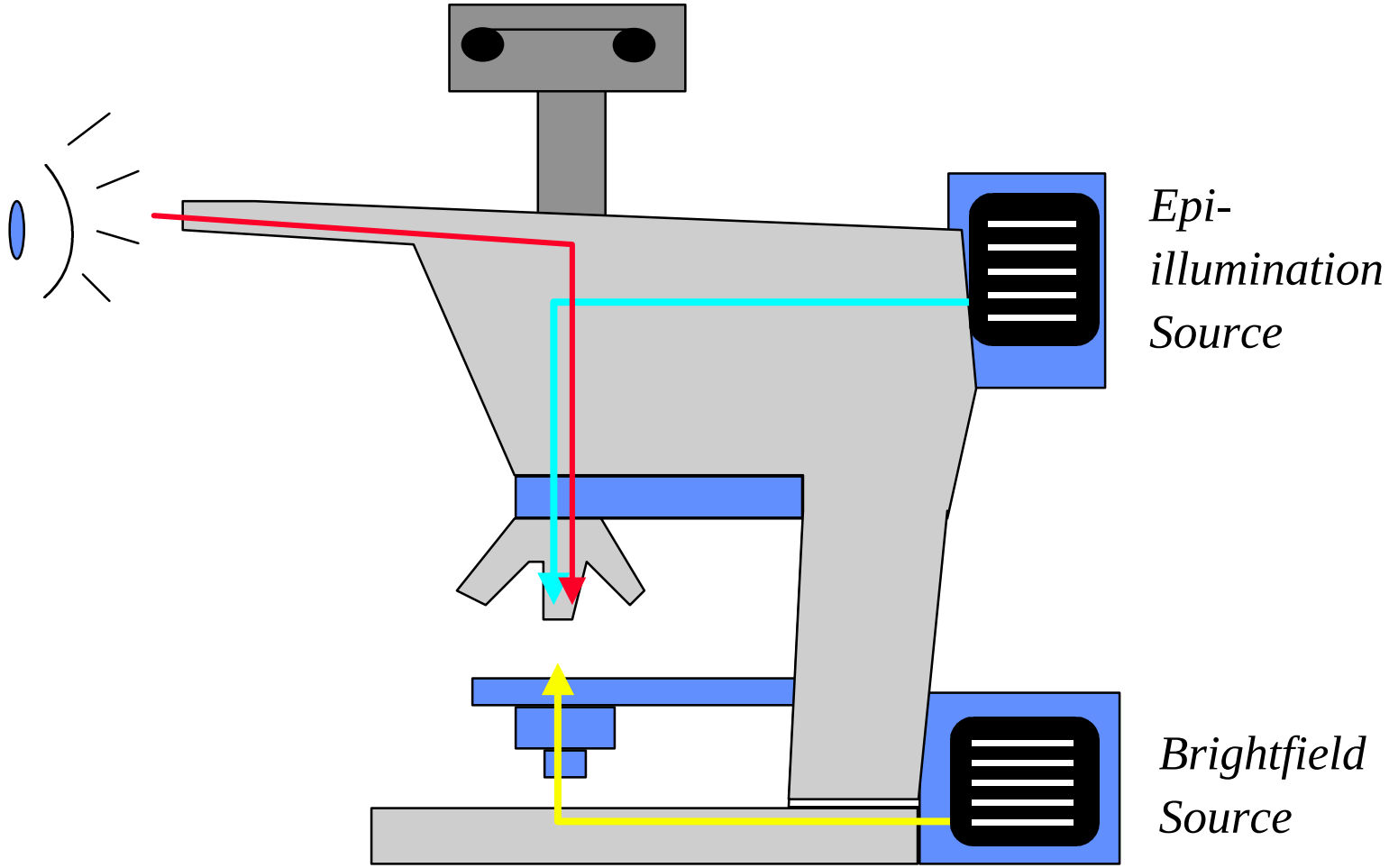


The Conventional Microscope

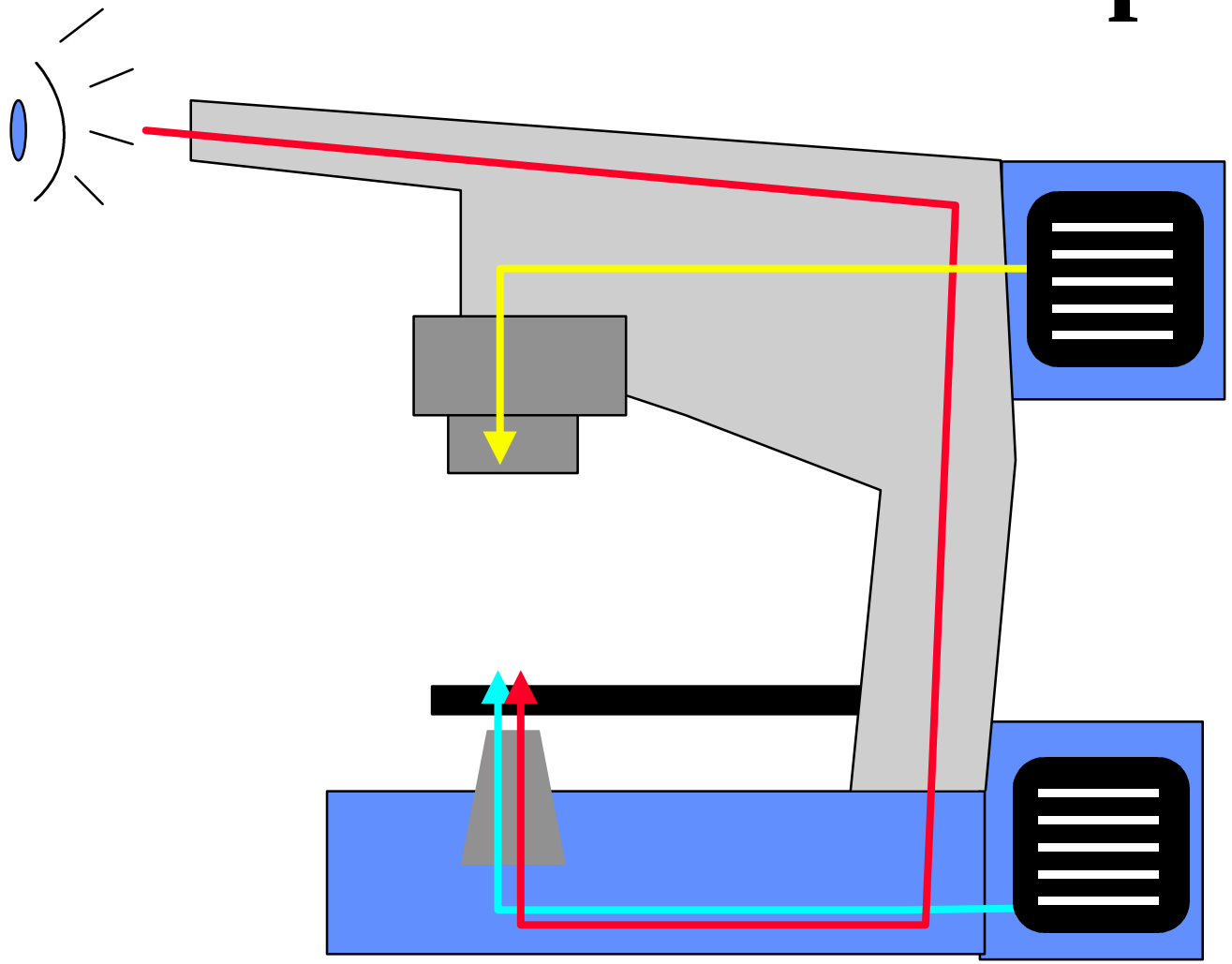


Modified from "Pawley "Handbook of Confocal Microscopy", Plenum Press

Upright Scope



Inverted Microscope

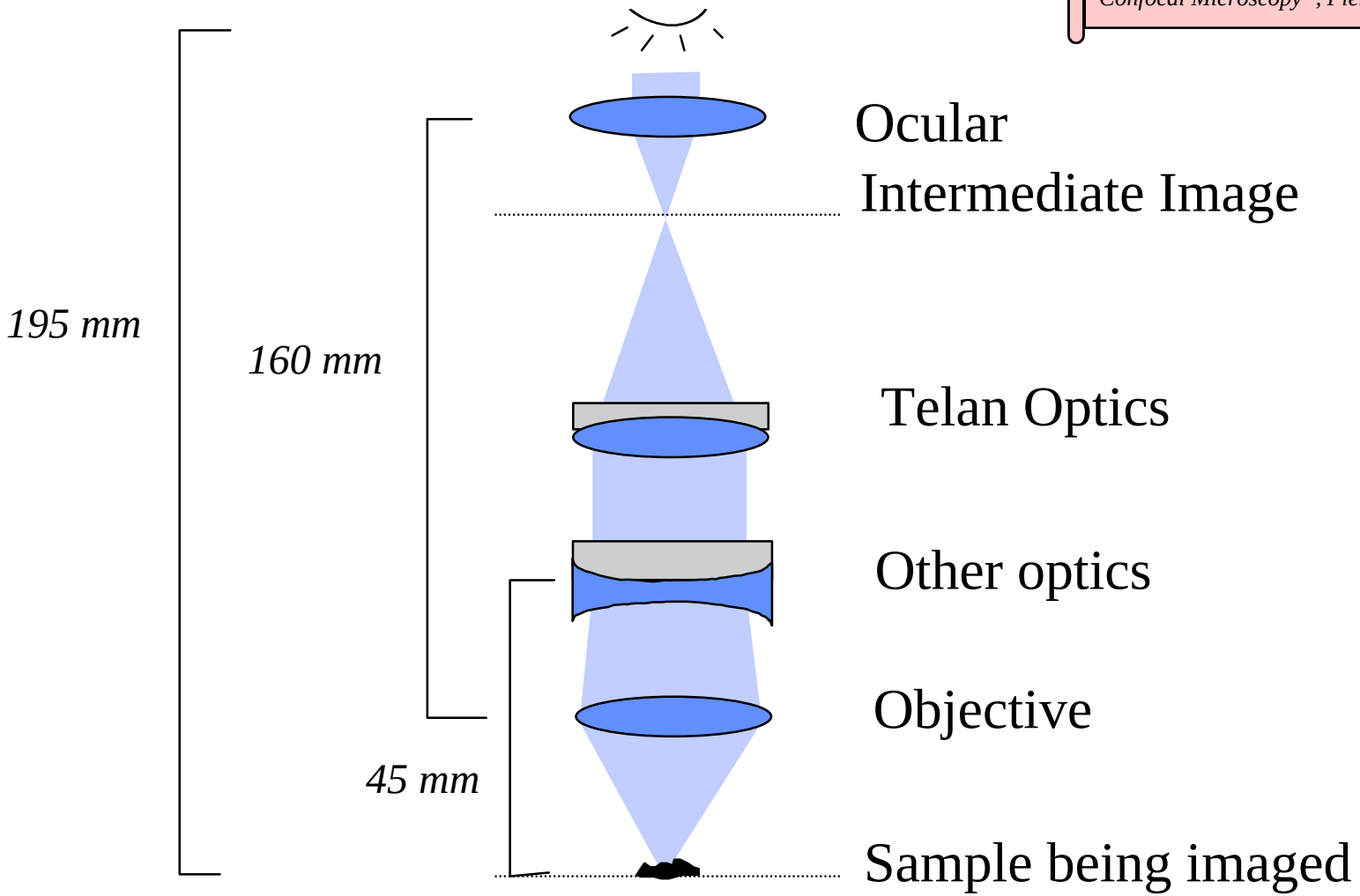


Brightfield Source

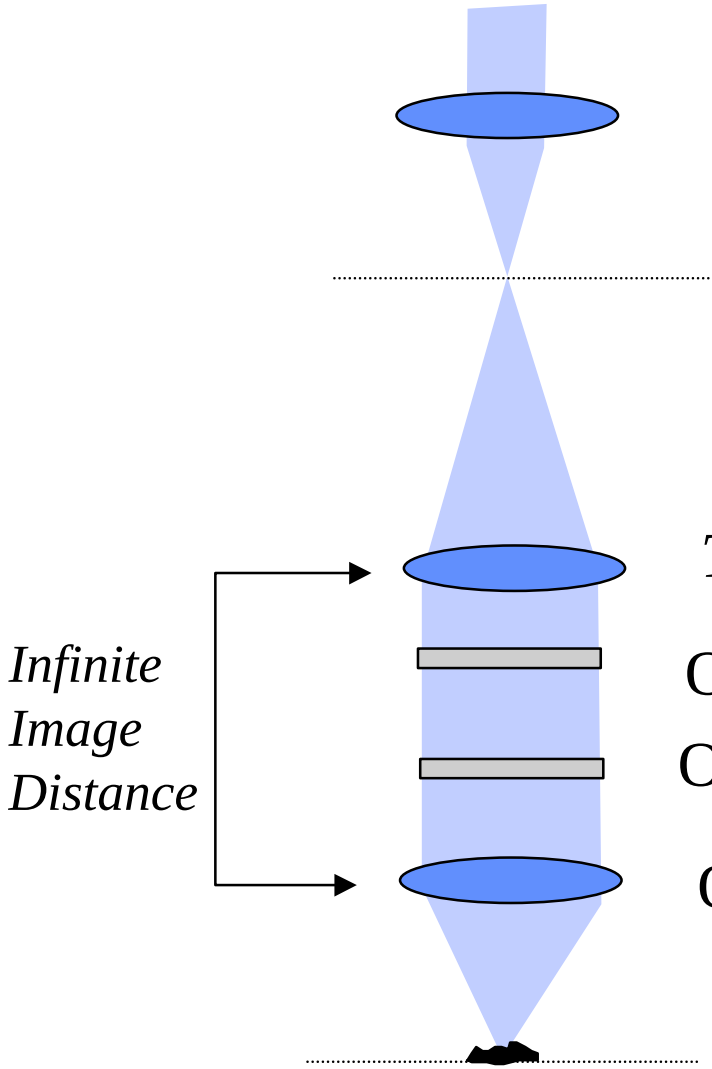
Epi-illumination Source

Conventional Finite Optics with Telan system

Modified from "Pawley "Handbook of Confocal Microscopy", Plenum Press



Infinity Optics



The main advantage of infinity corrected lens systems is the relative insensitivity to additional optics within the tube length. Secondly one can focus by moving the objective and not the specimen (stage)

Modified from "Pawley "Handbook of Confocal Microscopy", Plenum Press

Summary Lecture 1

- Simple versus compound microscopes
- Achromatic aberration
- Spherical aberration
- Köhler illumination
- Refraction, Absorption, dispersion, diffraction
- Magnification
- Upright and inverted microscopes
- Optical Designs - 160 mm and Infinity optics