Fundamentals of Light Microscopy

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Cochin - 682019

Light Microscope

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- **Microscope:** Instrument or a device that enables the user to see objects that are too small to be seen by the unaided eye.

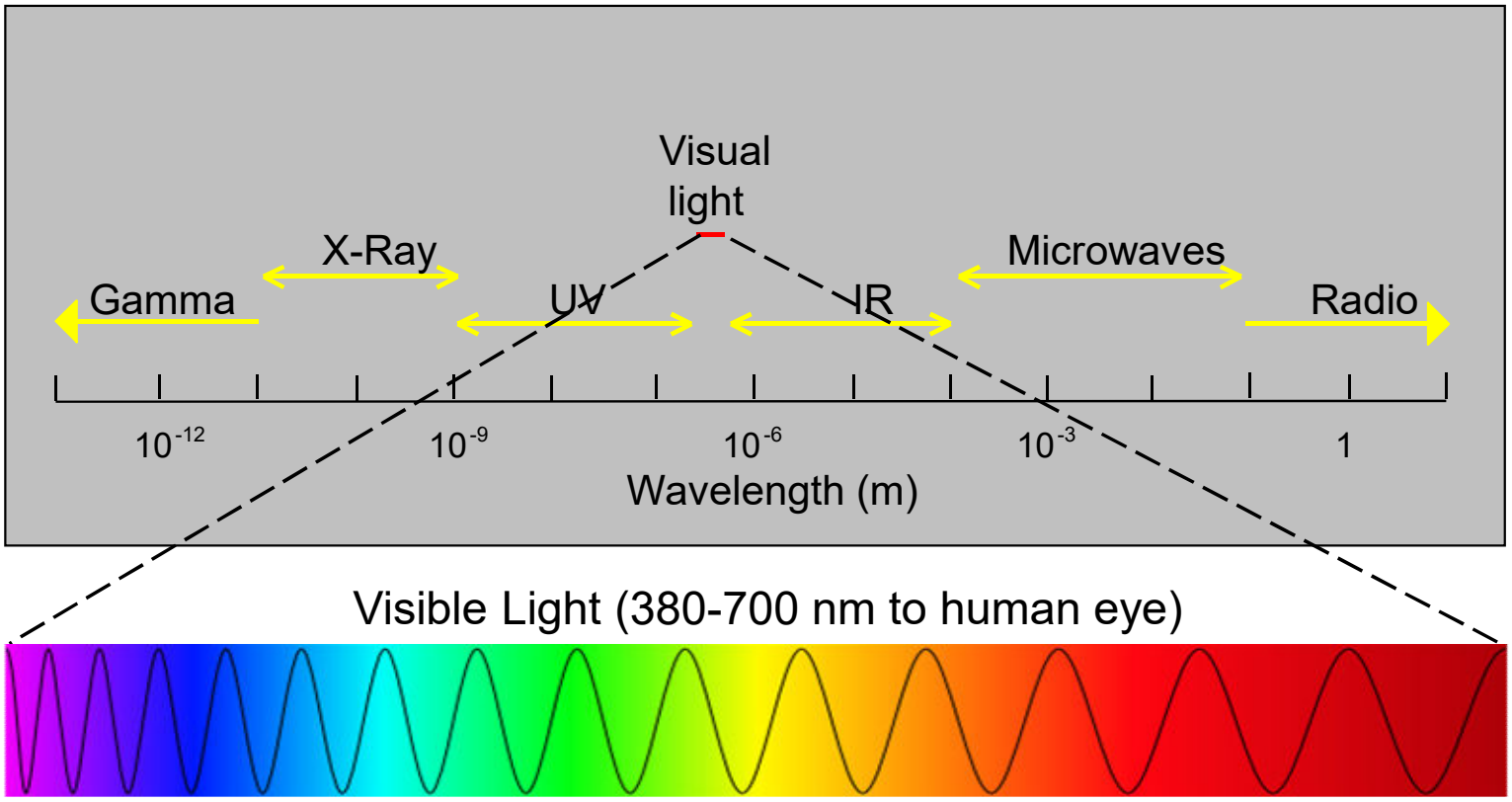
Greek :
mikros = small
skopien = to observe

- **Microscopy:** is the science of investigating small objects or structures using such device or instrument.

“Observation of small objects”

But “just” seeing the objects alone may not be enough!!!

Nature of Light - Electromagnetic Spectrum



"White Light" is the combination of all wavelengths originating from a source

Visible spectrum = light

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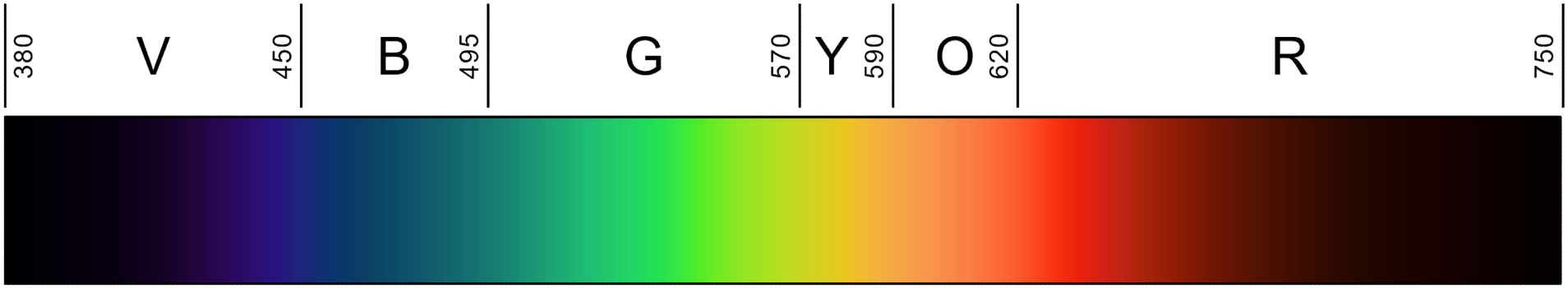

Blue = 0.4 X 10^{-6} m

Red = 0.7 x 10^{-6} m

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Light as a messenger

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- Light is the messenger and transports the object information from the specimen through the microscope
- Also translates the object information into microscopic **image** of the specimen
- The observer visualizes the **image of the specimen** and **never the specimen itself!**
- Only the **best management of light** allows translation of information as accurately as possible from object into an image which represents that image!

Nature of Light – dual property

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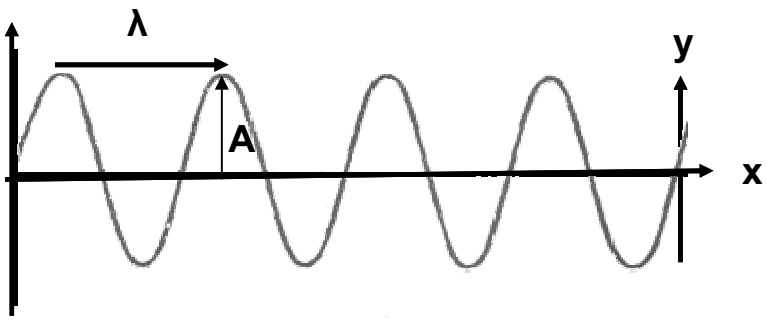
Wave nature of light

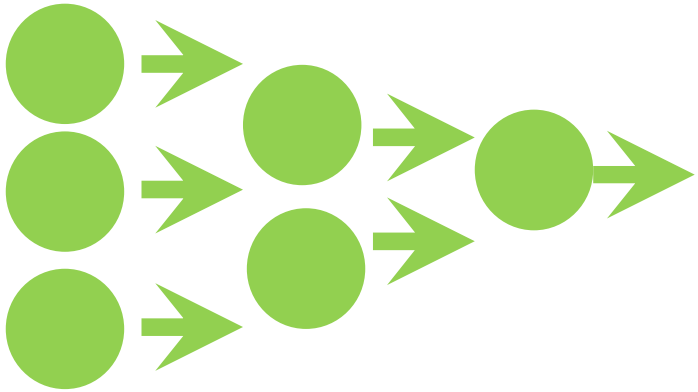
- ❑ Wave of energy, travelling through space
- ❑ Wave optics
- ❑ Image formation in microscope



- x = direction of travel
- y = direction of vibration
- A = amplitude
- λ= wavelength
- amplitude defines intensity of light
- wavelength defines content of energy and color:
  400 nm, high energy, blue
  550 nm, green
  750 nm, low energy, red

Particle nature of light

- ❑ Photons
- ❑ Geometric optics



Basic Properties of Light - Wavelength vs. Color

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Wavelength Range (nm)	Perceived Color
<400	Ultraviolet
400-430	Violet
430-465	Blue
465-500	Cyan
500-550	Green
550-580	Yellow
580-620	Orange
620-700	Red
>700	Near-Infrared Not visible to the human eye, but detectable by digital cameras (depending on model and wavelength).

Short wavelengths have more energy and cause more damage

Infrared wavelengths can induce molecular vibration, and heating

Light-Medium Interaction

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- Absorption
- Reflection
- Refraction
- Diffraction
- Dispersion

Light Interacts with Optics - Absorption

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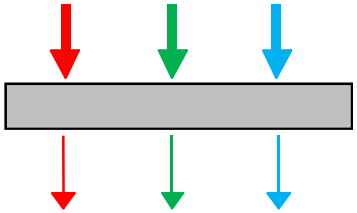
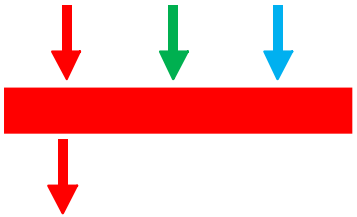
Reduction of all wavelengths



Transmission varies with wavelength

Blocking all but one wavelength (for instance red)



Medium illuminated by white light appears in color (for instance red)

Color filters, stained specimen

Light Interacts with Optics - Reflection

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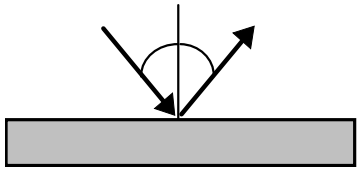
smooth surface
specular reflection
(mirror or glass)



- Direction of reflection predictable
- Incident angle of light = angle of reflection
- Mirror image
- Reflections are additive at each interacting surface
- Anti-Reflective coatings reduce reflection

Light Interacts with Optics - Refraction

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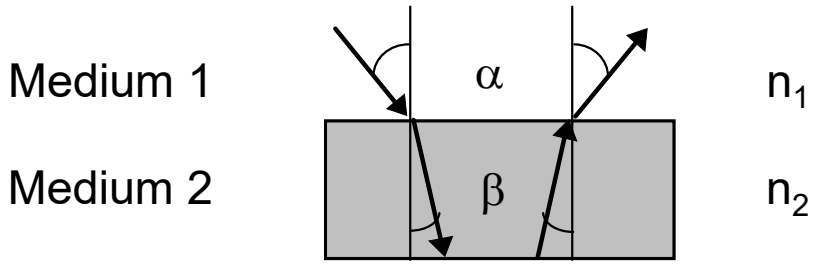
Change of travelling direction of light when passing from medium 1 to medium 2



- Refractive index **n** defines optical density of medium **(Vacuum, air: n=1, water: n=1.33, Oil: n=1.518, glass: n =1.52)**
- Higher optical density equals less velocity of light within the matter

Light Interacts with Optics - Dispersion

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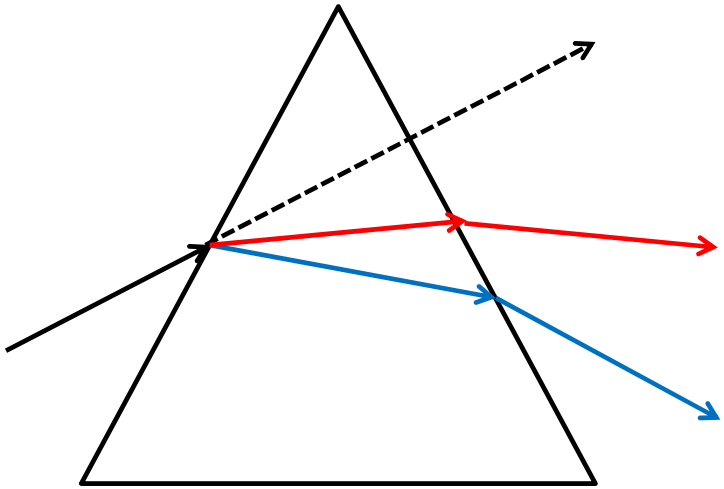
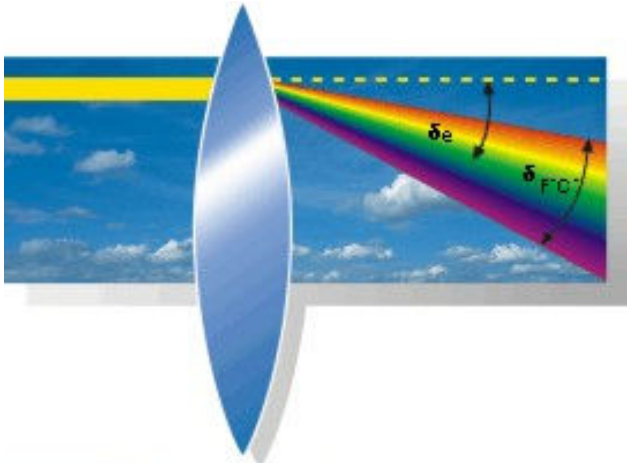
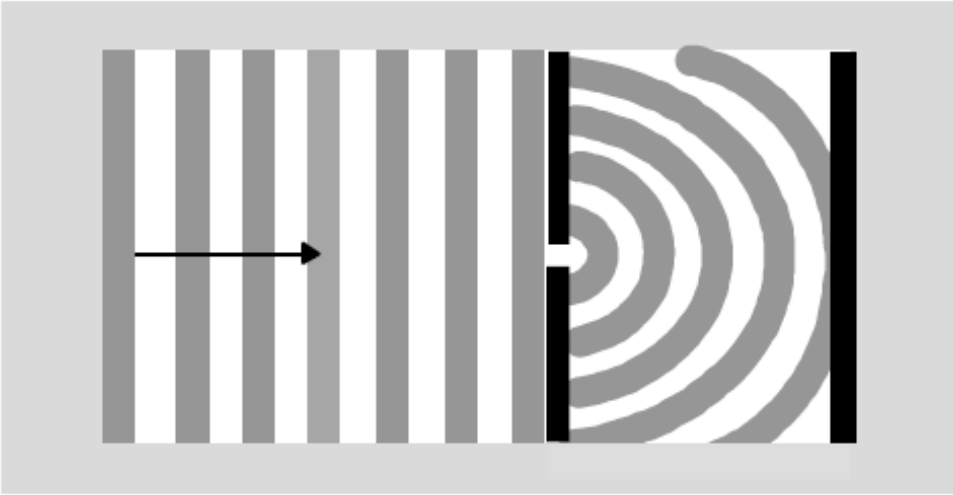
- Refractive index of medium depends on energy of light
- Light with higher content of energy (blue light) has higher angle of refraction than light with lower content of energy (red light)
- Dispersion of white light into colors of rainbow when travelling through a prism
- Dispersion causes **chromatic aberration** in microscopy

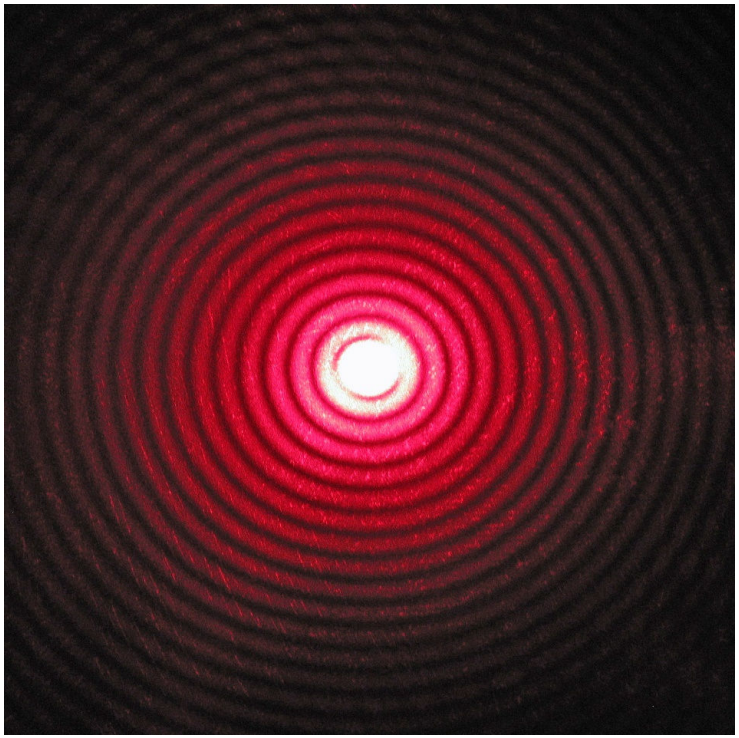




Light Interacts with Optics - Diffraction

- Bending of light when it passes an edge or bending of waves around a corner.
- Also occurs while a wave encounters an obstacle or a slit or grid pattern.
- Crucial for the understanding of resolution.
- **Diffraction and interference** are important for image formation





https://en.wikipedia.org/wiki/Diffraction

Image Formation-1

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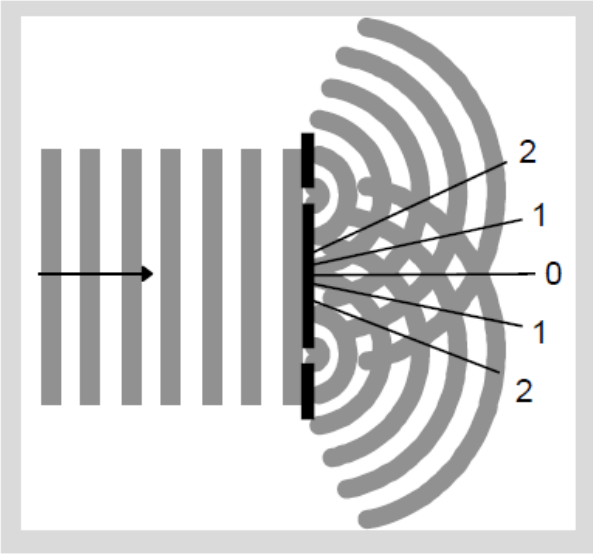
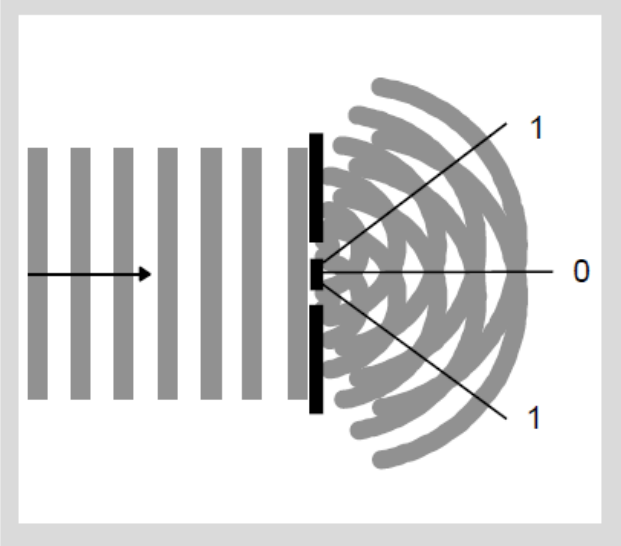
Diffraction at 2 gaps which are wide apart



Interference of semi-circular waves causes maxima and minima of diffraction. Maxima of diffraction close to each other

Diffraction at 2 gaps which are close to each other



Interference of semi-circular waves causes maxima and minima of diffraction. Maxima of diffraction wide apart

Image Formation-2

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- Details of the specimen diffract light.
- **Smaller the details** of the specimen are the **more the light is diffracted.**
- Diffracted light carries information about the specimen details
- Diffraction maxima are separated by diffraction minima
- Maximum in center = 0^{th} Order maximum = **principal maximum= non diffracted light**
- Adjacent maxima = 1^{st} order, 2^{nd} order maxima =**secondary maxima = diffracted light**
- Size of structures on sample determine distance between maxima

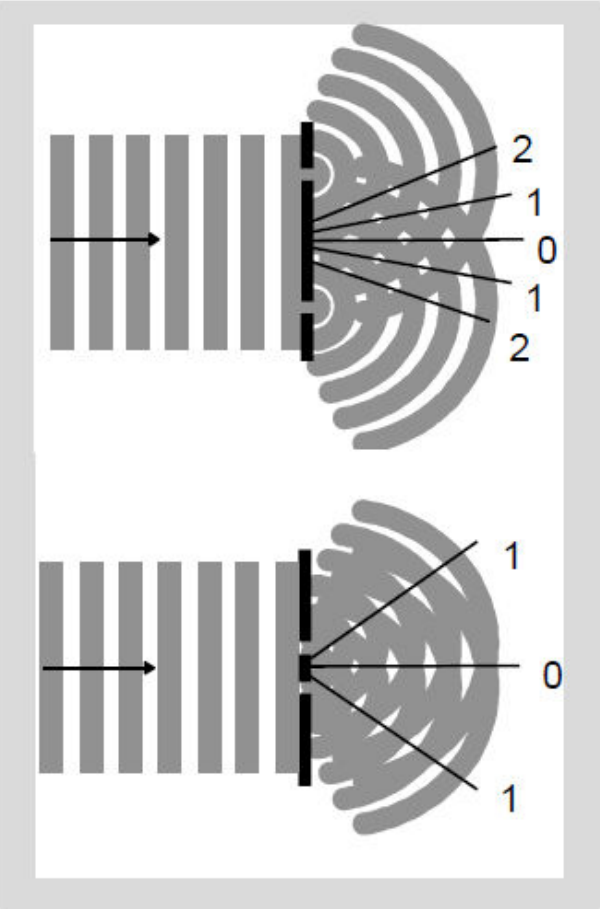

Image Formation-3

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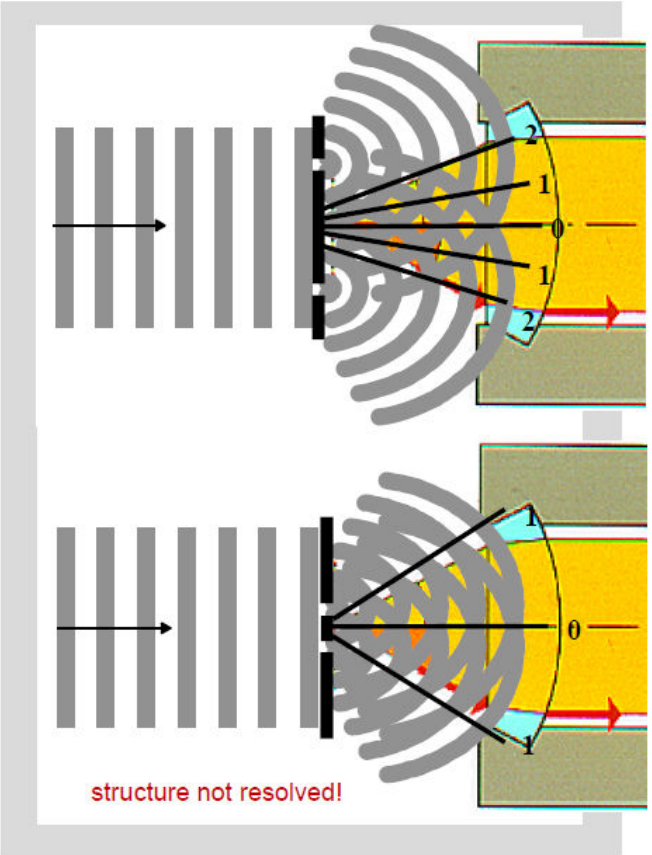
- Diffraction pattern contains information about diffracting structure
- Intermediate image is formed through interference of diffracted light (at least 1st order maximum)
- Capturing of diffracted light by objective is necessary for image formation, i.e. **RESOLUTION** of details
- Objectives with a **wide opening angle** observe a wide angle in space and are able to capture more of the diffracted light than objectives with small opening angle



Microscope – preconditions

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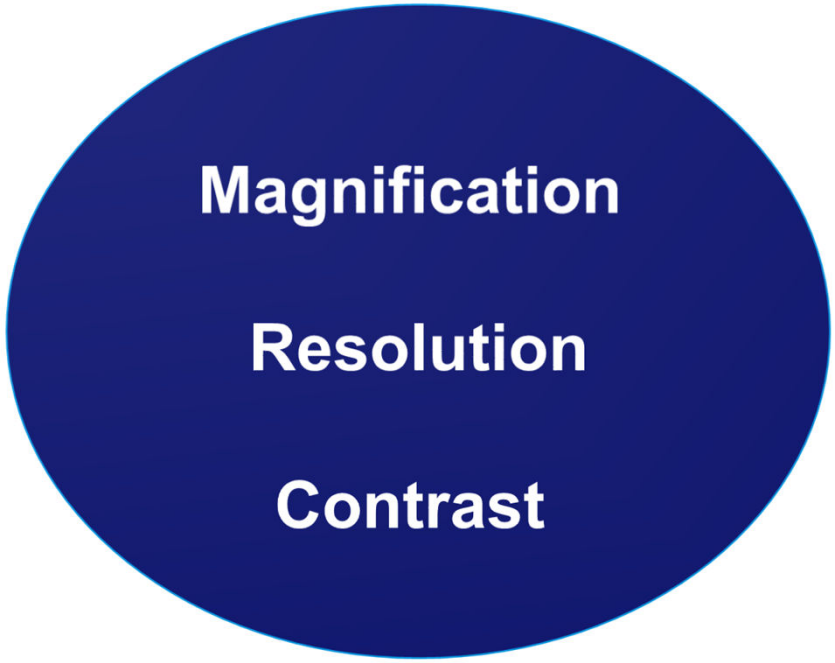
- To observe “small objects” 3 preconditions needs to be fulfilled



- Only fulfillment of these 3 preconditions allows translation of information as accurately as possible from object into an image which represents that object.

Magnification

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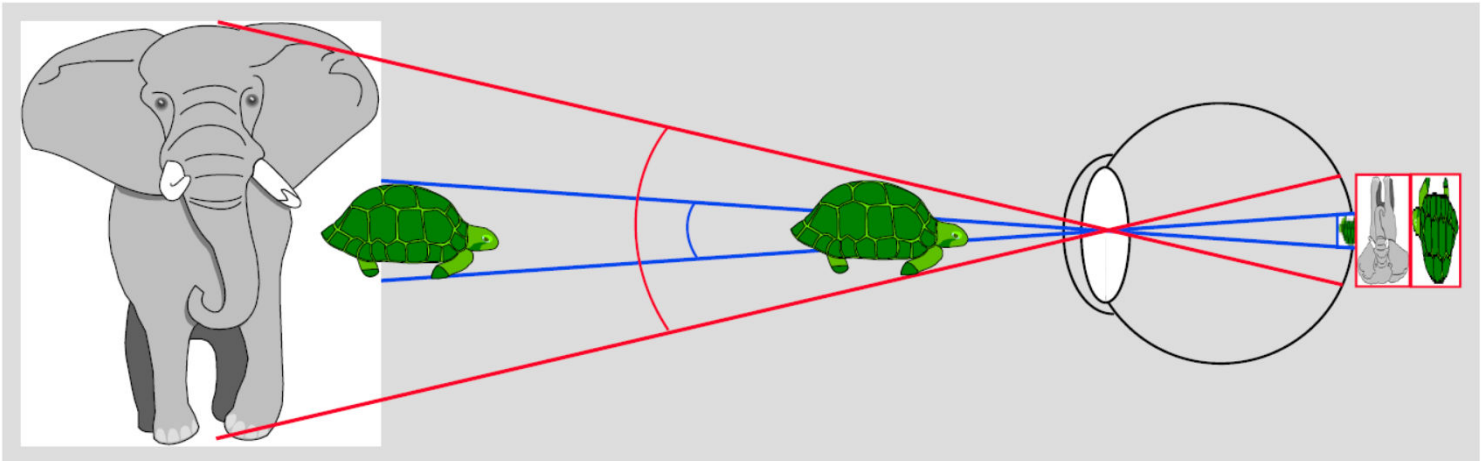
- In order to see small objects with the eye small objects have to be **magnified** to an appropriate size.
- angle formed by light rays from an object which enter the eye
- the bigger the object, the wider the viewing angle, the more details can be recognized and vice versa.
- the bigger the viewing angle the more distinct the object appears
- more details to investigate ➔ increase viewing angle



Magnification

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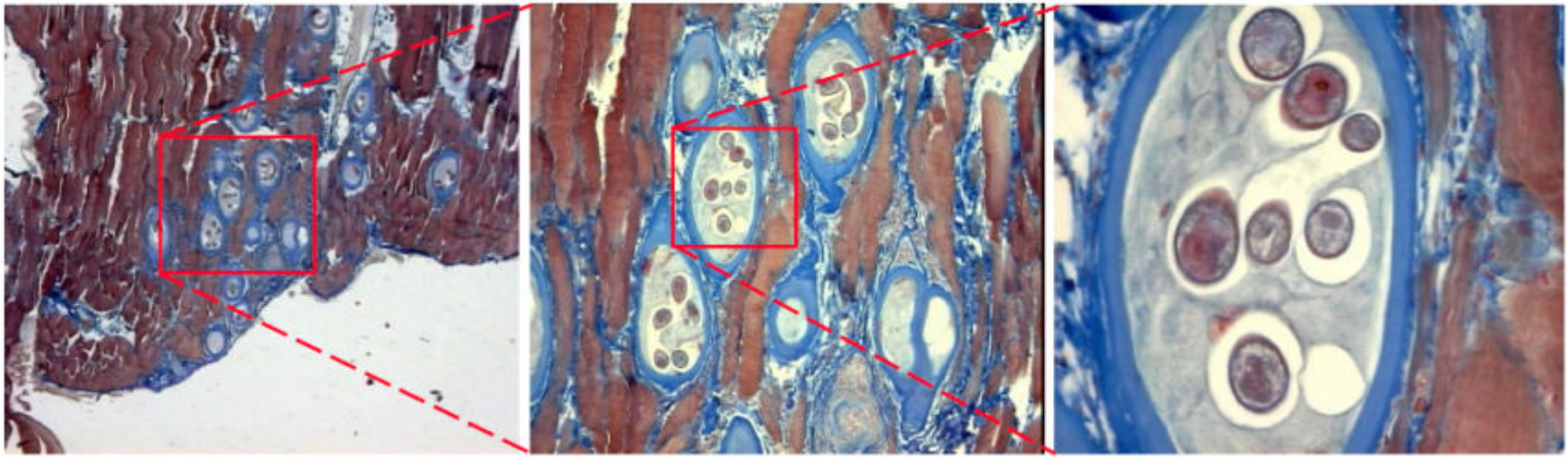
In order to see small objects with the eye the images of these objects have to be **magnified** to a appropriate size



Useful Magnification

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- Magnification of specimen details has to be adapted to limiting resolution of the eye. i.e. smallest details resolved by the objective have to be offered to the eye between 200µm to 400µm in size.
- Higher magnification might cause empty magnification
- Lower magnification might not be detectable by eye

Rule of Thumb:
500 x N.A – 1000 x N.A = Useful magnification

10x/0.25 Objective
(500 x 0.25 – 1000x0.25) = 125x to 250x

100x/1.4 Oil Objective
(500 x 1.4 – 1000 x 1.4) = 700x to 1400x

Useful & empty Magnification

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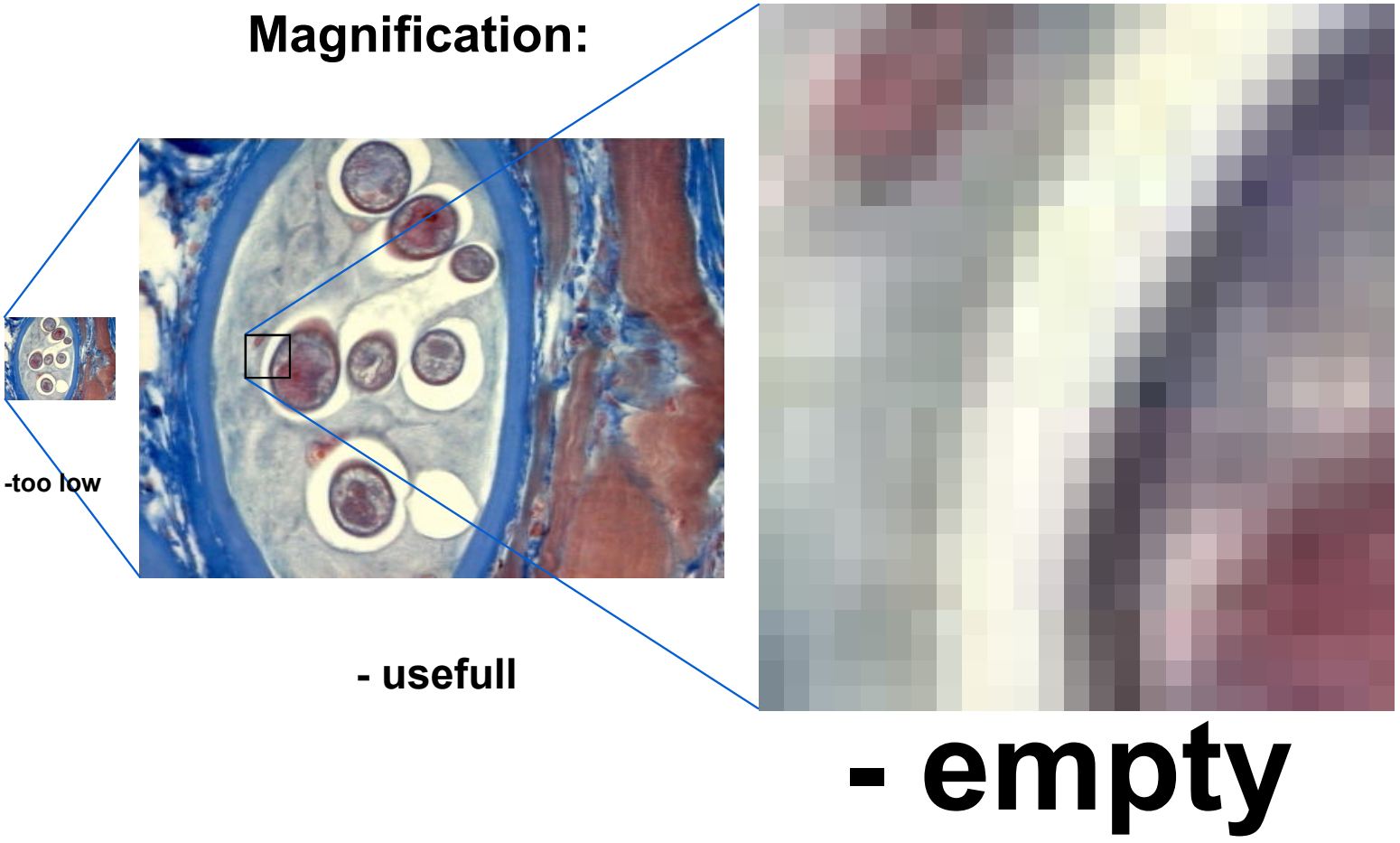

Magnification - components

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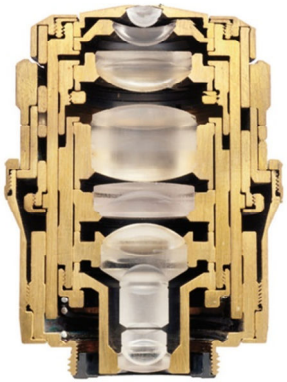
Objectives



Eyepieces

Microscope Eyepiece (Ocular) Anatomy

Total Magnification = Objective magnification X eyepiece magnification

Resolution

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- The smallest distance between to objects up to which the two objects can still be seen as two separate objects.

$$d_0 = \frac{1.22 \cdot \lambda}{N.A._{Objective} + N.A._{Condenser}}$$

- Resolution in microscopy can be categorized as

Spatial – (in space or distance)

- **Lateral (XY) resolution** : distance between two objects that are in the same focal plane.
- **Axial (XZ, YZ) resolution:** depth wise or resolution along the axis

Temporal (in Time, speed) e.g. 20 frames per second

Resolution – N.A. (numerical aperture)

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$$NA = n \cdot \sin \alpha$$

n = refractive index

n = speed of light in space / speed of light in substance

e.g. n water 1.33 = 300,000 / 225,000

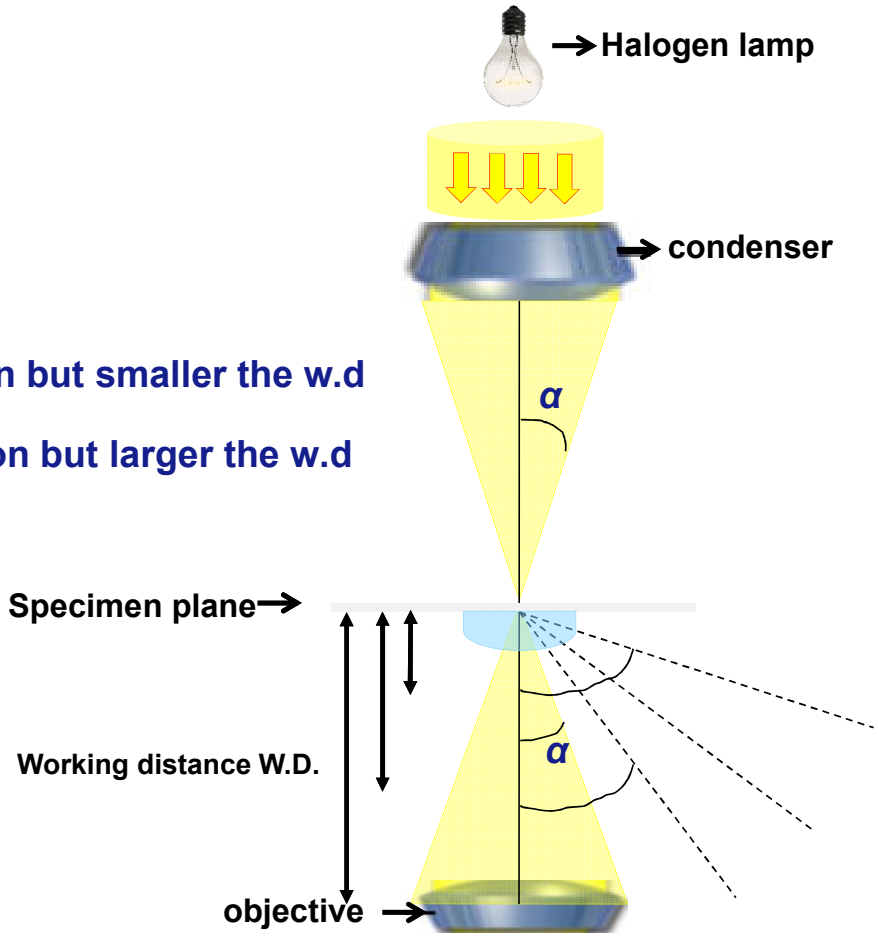
Larger the N.A higher the resolution but smaller the w.d

Smaller the N.A lesser the resolution but larger the w.d

Air = 1
Water = 1.33
Oil= 1.518

Remember the N.A of the objective will never match the refractive index of the immersion medium

If Objective N.A < 1 it will be dry objective
If Objective N.A > 1 ≤ 1.2 it will be water immersion
If Objecive N.A ≥ 1.25 it will be oil immersion



Microscope - classifications

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- Simple Microscope
- Compound Microscope
- Stereo microscope
- Zoom microscope

→ Type & number of lenses used

- Upright Microscope
- Inverted Microscope

→ Position of the objective/objective nosepiece

- Bright field microscope
- Phase contrast microscope
- DIC microscope
- Darkfield microscope
- Fluorescence microscope

→ Contrast Techniques

- Confocal microscope
- Super resolution microscope

→ Principle and resolution

- Widefield microscope
- Scanning/optical sectioning microscope

- Imaging devices
- Closed/box type systems

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UPRIGHT MICROSCOPE

INVERTED MICROSCOPE

STEREO MICROSCOPE

Upright vs Inverted microscope

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Objective

Components of a typical microscope

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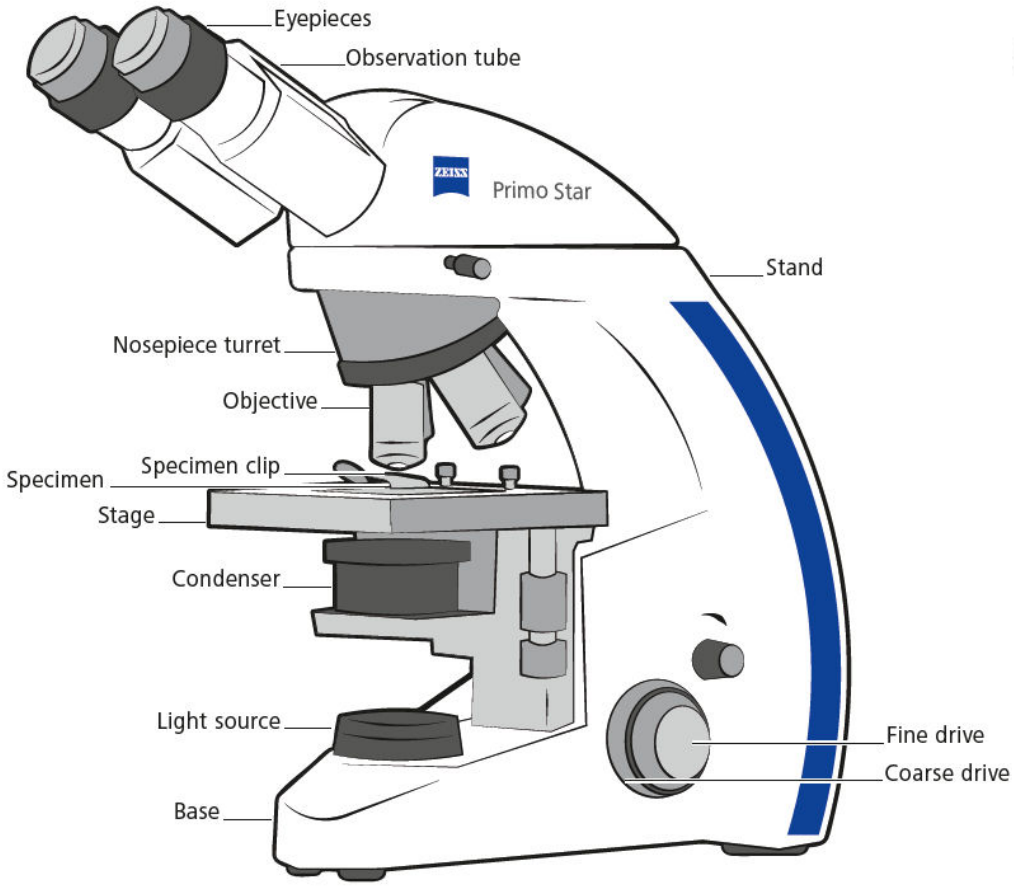

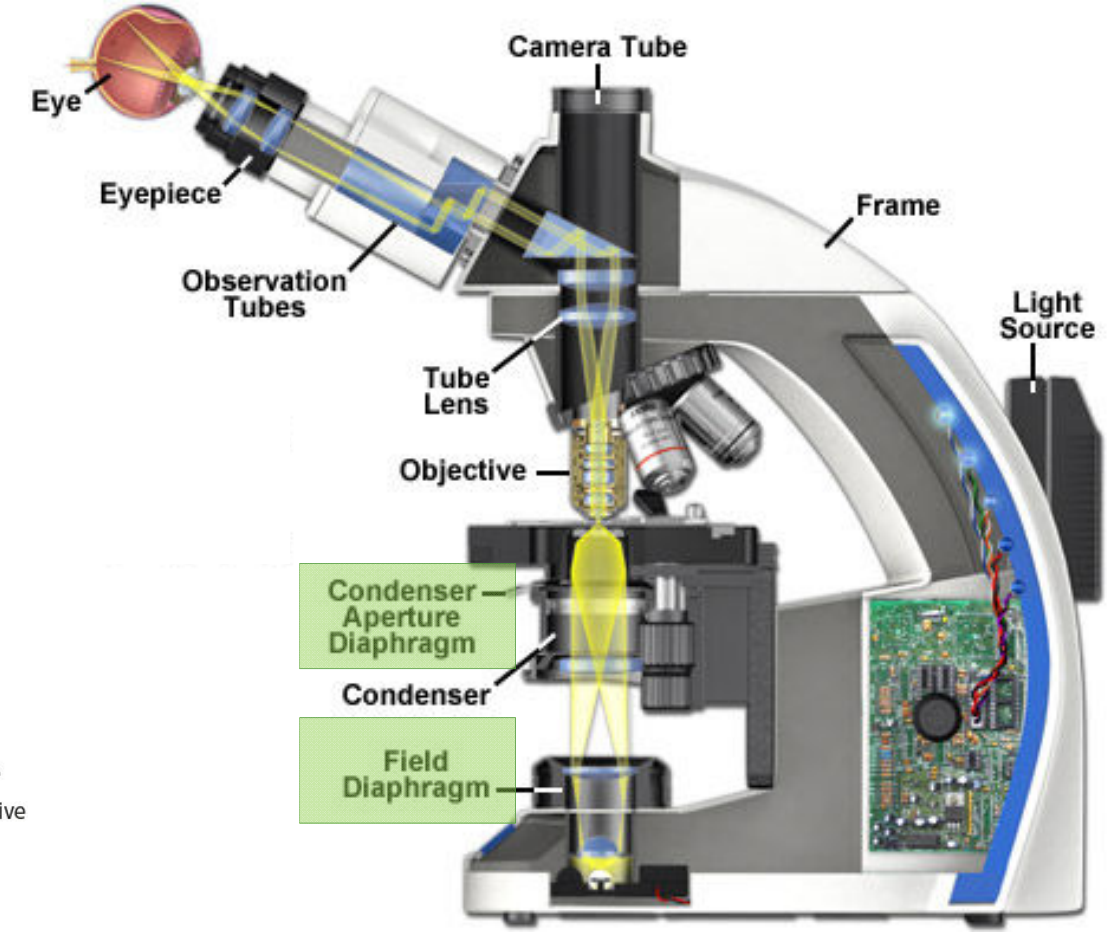

Components of a inverted microscope

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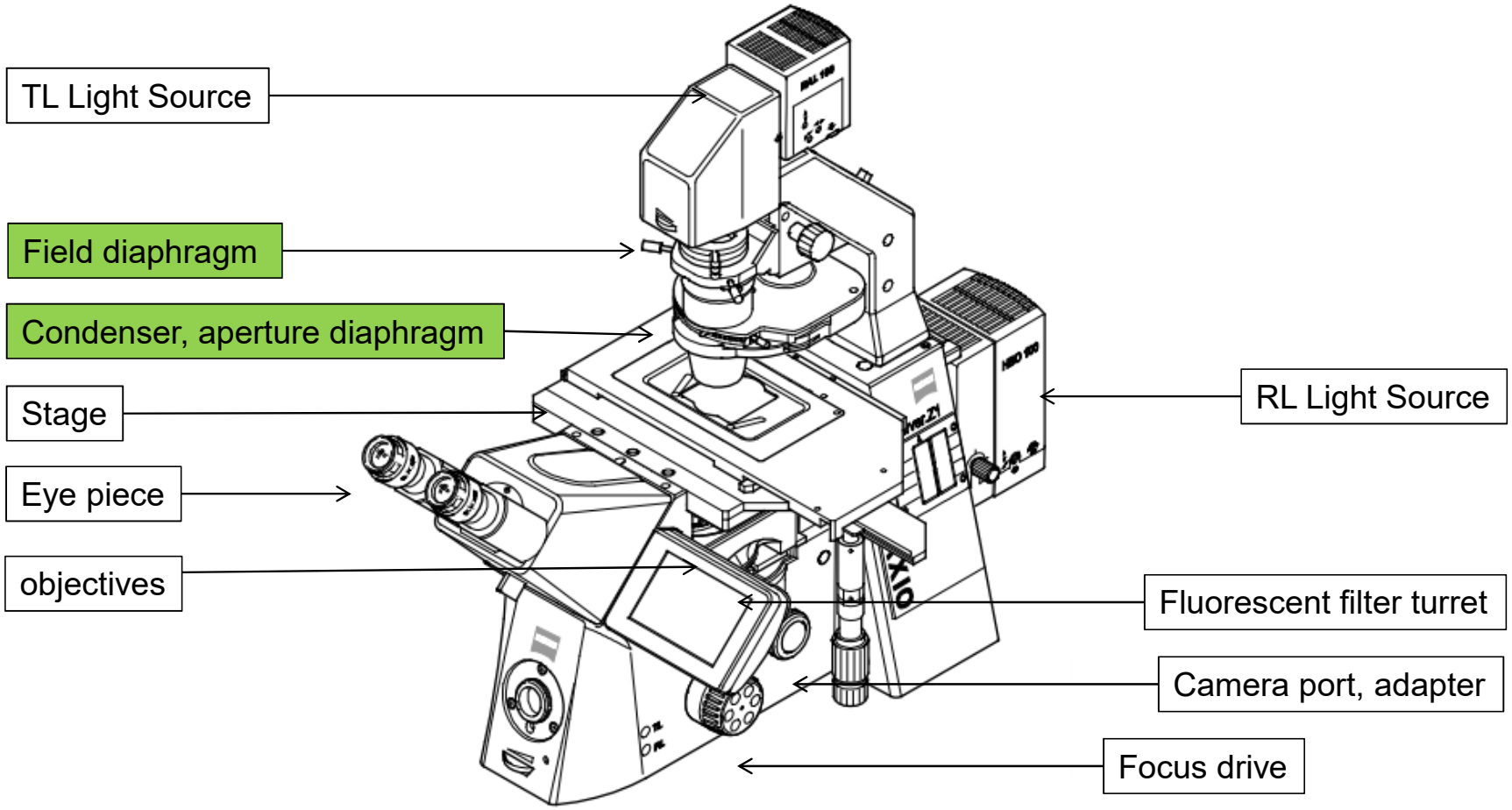

Components – Field and Aperture diaphragm

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Field Diaphragm :

- Controls the field or area of illumination of the sample.
- Useful for highly photo-sensitive sample
- Typically located under the condenser and not a part of the condenser.

Field and aperture diaphragm function

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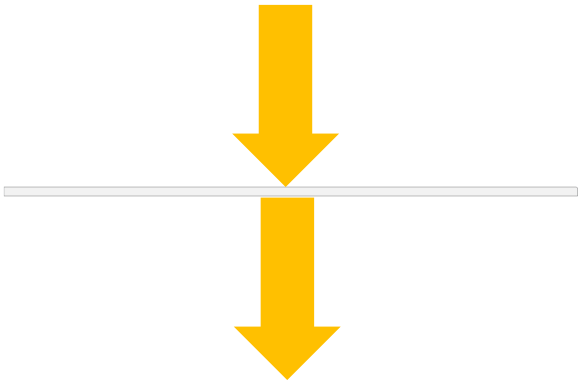
Aperture Diaphragm :

- Controls the amount of light that passes through lens and affects the intensity.
- Controls the N.A of the condenser lens.
- Typically is part of the condenser.

Components – Light Sources

Transmitted light:

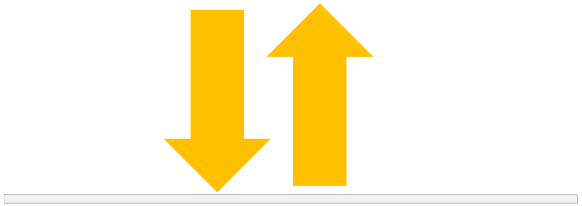
- ❑ **Halogen lamp**
- ❑ **LED**



- ❑ **Brightfield**
- ❑ **Phase contrast**
- ❑ **DIC**
- ❑ **Darkfield**

Reflected light:

- ❑ **Mercury arc Lamp**
- ❑ **Metal Halide Lamp**
- ❑ **LED**
- ❑ **LASER**



- ❑ **Fluorescence**

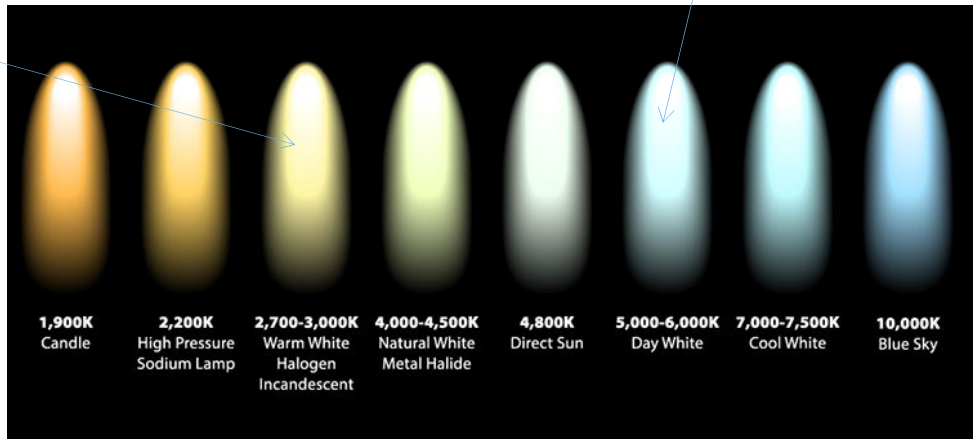
TL is unidirectional path and RL is bidirectional path

TL - Light Source

Halogen

LED

Color temperatures:



LED Benefits

- Bright enough for all applications
- Even illumination
- Stable over time
- Long life

Light Sources - FL

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HBO (mercury arc):

- ❑ least expensive solution, life time approx. 300-350 hrs
- ❑ but cannot be used for fast imaging, (reflector turret)
- ❑ no trigger control at the light source level
- ❑ Intensity cannot be regulated at source level , need attenuators to control intensity
- ❑ Requires alignment

HXP 120 (metal halide) :

- ❑ More expensive than HBO 100
- ❑ Longer life time approx. 2000 hrs, no alignment required
- ❑ Trigger control possible
- ❑ Intensity and shutter control possible through software

(LED):

- ❑ Relatively cheaper as a whole package (light source, reflector modules, filters etc)
- ❑ Guaranteed lifetime of at least 15,000 hrs
- ❑ Extremely fast switching between LEDs (< 10ms)
- ❑ Ideally suitable for fast live cell imaging (multichannel)

RL – Fluorescence light sources

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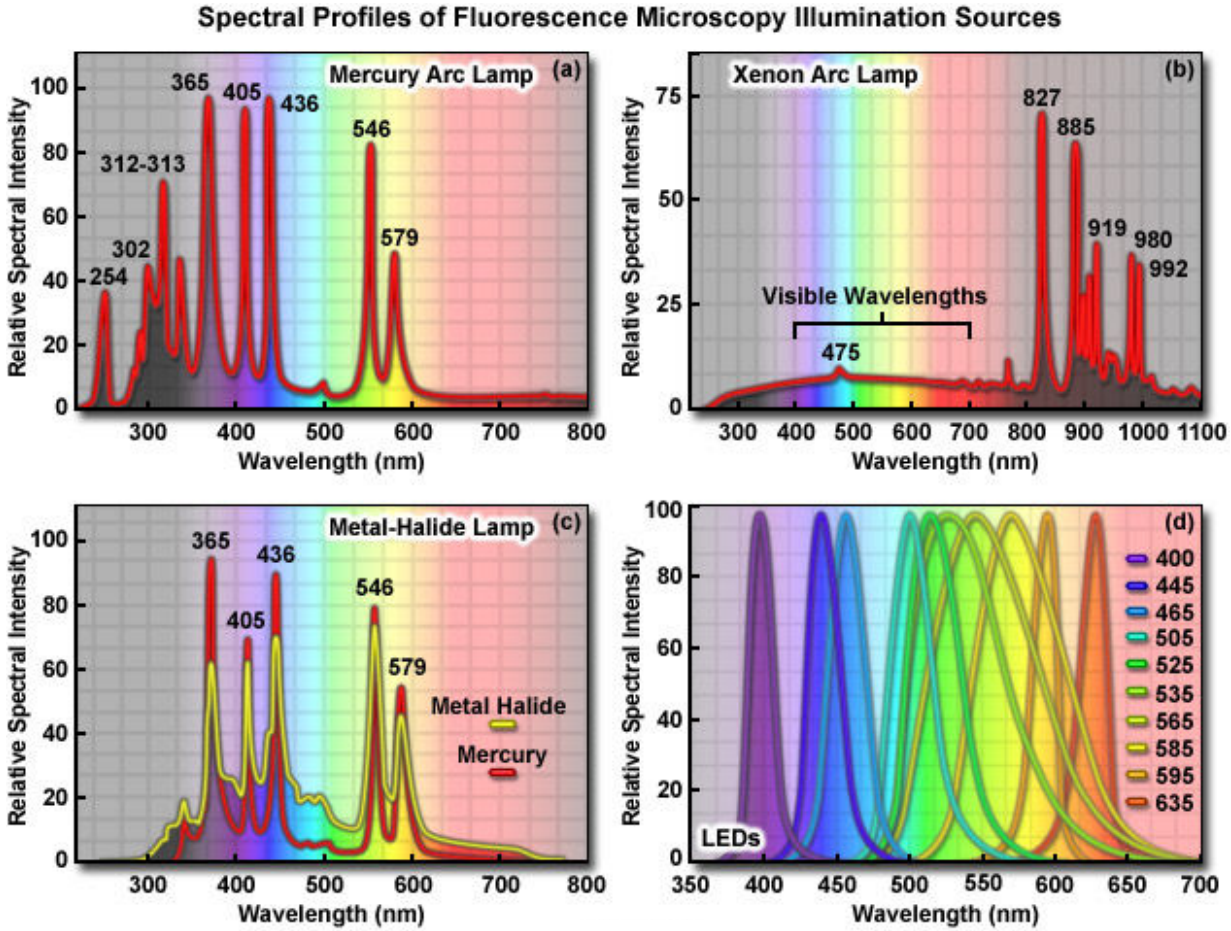
- ❑Mercury arc lamp (HBO)
- ❑Metal halide lamp (HXP)
- ❑Xenon lamp (XBO)
- ❑LEDs



Light source for fluorescent microscopy - Light Emitting Diode (LED)

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- Emit a discrete wavelength or narrow band
- High stability for better quantification
- **Microsecond switching on/off, ideal for live cell imaging**
- No need for fast shutters or excitation filter wheels
  – No vibrations
- Thousands of hours of usage
- Lower power consumption
- Less heat produced

LEDs are quickly replacing white light sources!

Geometric beam paths – Conjugate planes

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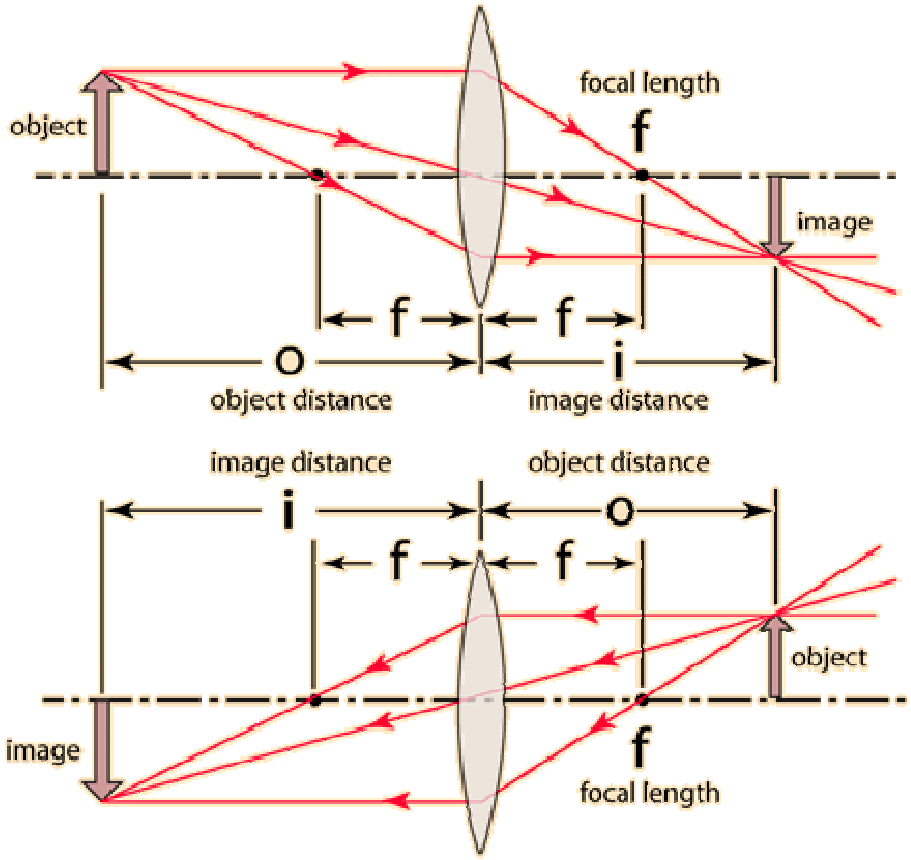
- **Conjugate planes:** The position of an object and its image are interchangeable.
- Similarly there can be two different optical components at different positions but will still create their image at a common point.
- e.g. the field diaphragm and the specimen plane of modern microscope creates the image on same plane.
- There are two sets of conjugate planes in a microscope.
  - **Field stop set of conjugate planes** (image forming beam path)
  - **Aperture stop set of conjugate planes** (illuminating beam path)



http://hyperphysics.phy-astr.gsu.edu/hbase/geoopt/conjug.html

Prof. August Köhler

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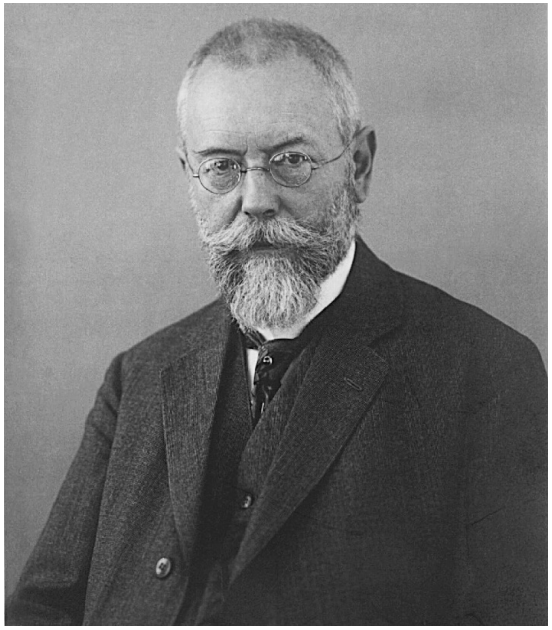

Prof. August Köhler
(1866 – 1948)

- Developed two beam path concept in microscope
- Beam path dedicated for illumination of the specimen.
- Beam path dedicate for image formation.
- To ensure a homogenous illumination of the specimen through precise positioning of filed diaphragm and condenser.
- Optimal adjustment of contrast techniques and resolution through aperture diaphragm and condenser.

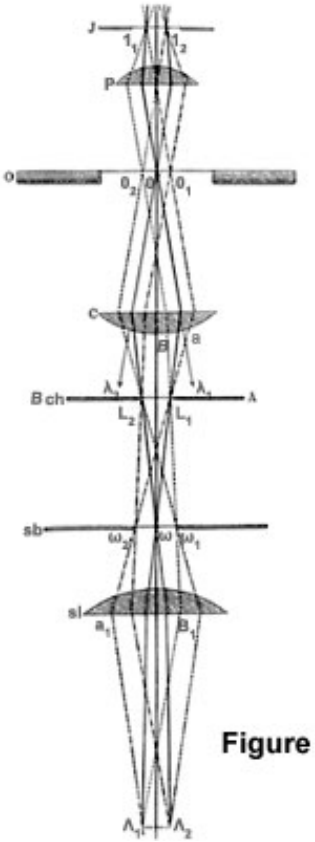

Figure 1

Steps to Köhler illumination

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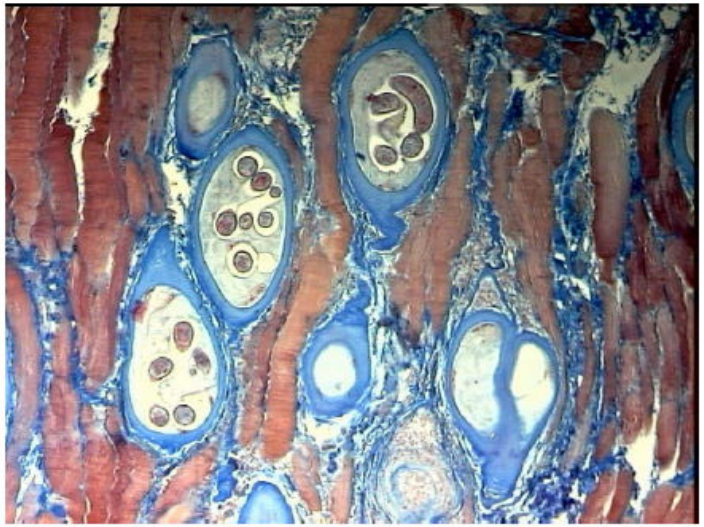
- Move condenser to the upper position, switch in front lens, set condenser turret for brightfield.
- Choose 10X magnification

- Focus the specimen using fine focus drive



Steps to Köhler illumination

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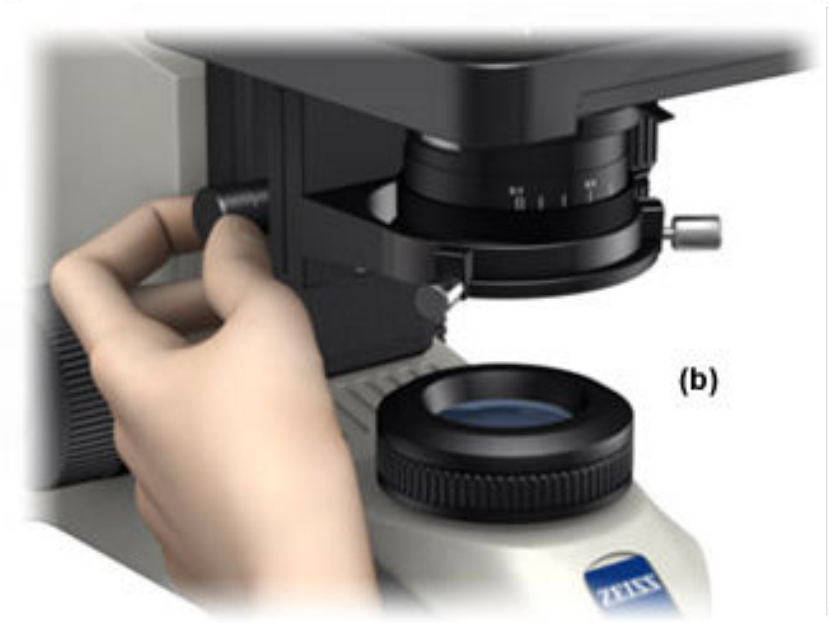
- Close the field stop until diaphragm is visible

- Focus the field stop by moving the condenser up or down



Steps to Köhler illumination

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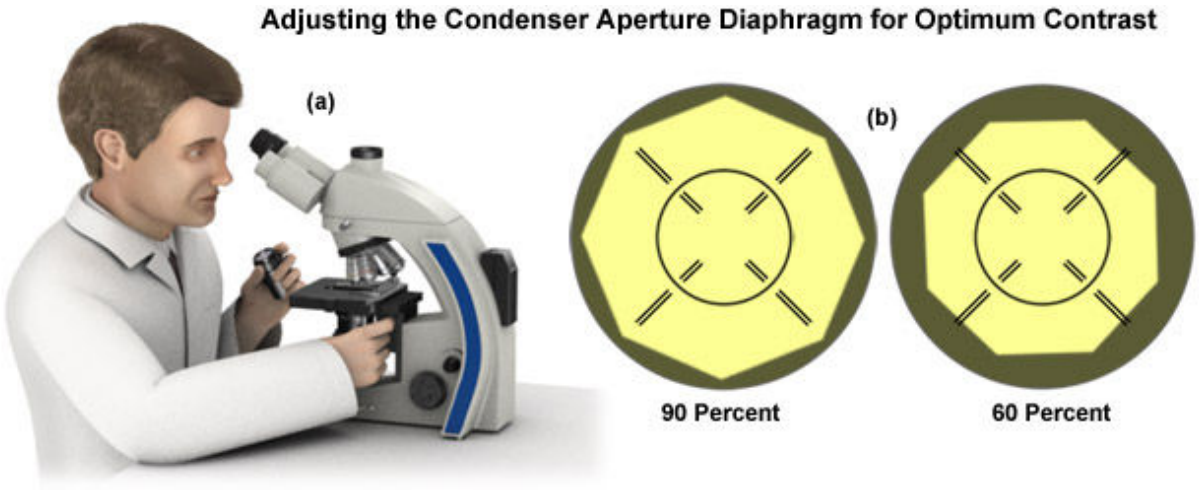
- Centre the condenser to center the field stop

- Open the field stop until the iris disappears out of the view

Steps to Köhler illumination

- Remove the eyepiece from the tube and look into the tube, close the visible aperture stop until 3/4th of the visible opening of the objective (pupil of objective) is illuminated



Microscope Objectives

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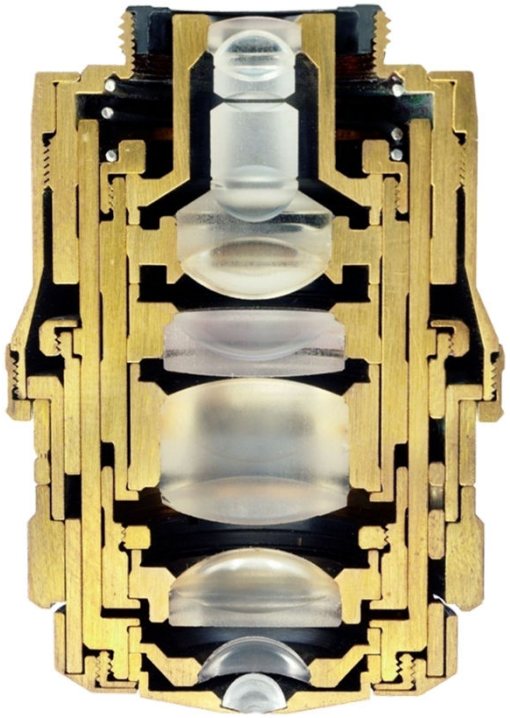
- Numerical Aperture
- Immersion choices
- Magnification
- Transmission UV/Vis/IR
- Chromatic Correction
- Optical Correction
- Working Distance



Example: Plan-Apochromat 100x/1.30 oil

Microscope Objectives

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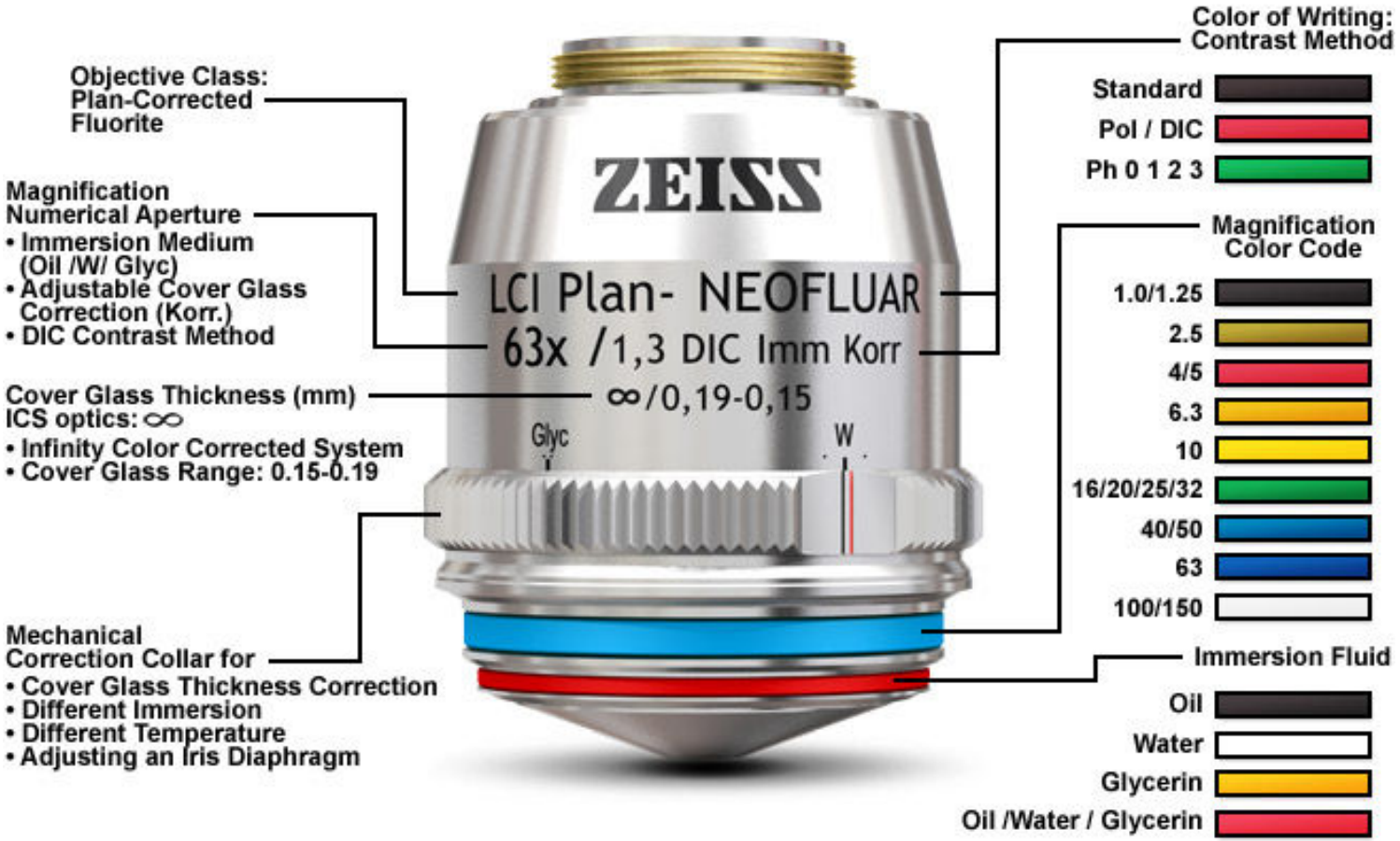
Deciphering Microscope Objective Specifications



Objective - Image Corrections

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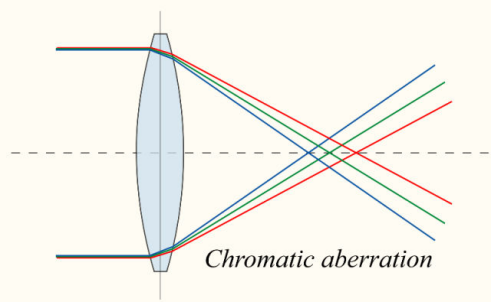
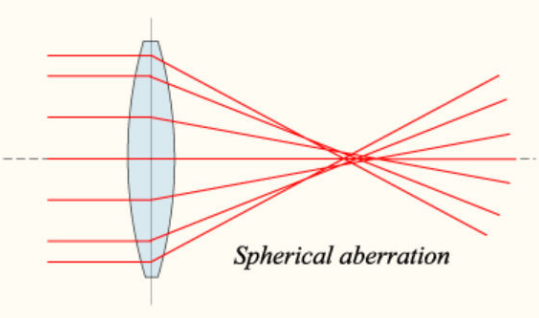
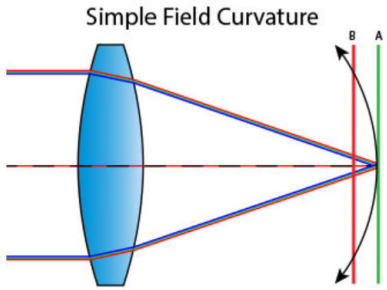
Image Defect	Cause	Solution
Different focal point for different wavelength	Chromatic Aberration	Achromatic lens Different grades of correction
Different focal point for different distance to the optic axis	Spherical Aberration	Aspherical lens High NA requires fixed cover glass thickness and immersion/embedding medium
Curved image surface	Field Curvature	Optimized flat-field correction All lens except Fluar







Objective - Comparison of common types

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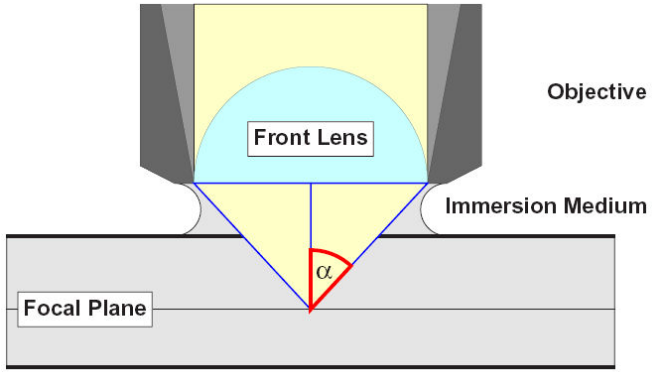
Simple summary	Application	Color correction	Flatness	Transmission
Entry level	Routine	Achromatic	20-23	380-1000
All round	Universal	Semi-Apo	25	340-800
High transmission	Fluorescence	Achromatic	no	340-800
High performance	High resolution	Apochromatic	25	340-800

10x objectives:

Objective	NA	WD	FOV	Flatness	Color corr	UV trans	IR trans
Achromatic	0.25	4.5	23	++	++	+	++
EC PN	0.3	5.2	25	++++	++++	++++	+++
Fluar	0.5	1.6	23	+	+	+++++	++++
Plan-Apo	0.45	2	25	+++++	+++++	+++	++++

Microscope objective - Resolution and Numerical Aperture



$$d = 0.61\frac{\lambda}{NA}$$

$$NA = n \cdot \sin\alpha$$

The resolution limit is reached, when two point-like objects can not be imaged as two distinct structures anymore.

The distance between the objects is called the resolution limit.

Smaller wavelengths produce better resolution.

d	resolution
λ	wavelength
NA	numerical aperture
α	opening angle
n	refractive index

Objective Choice - Light Throughput

inBiotek Microsystems

Light Collection Efficiency

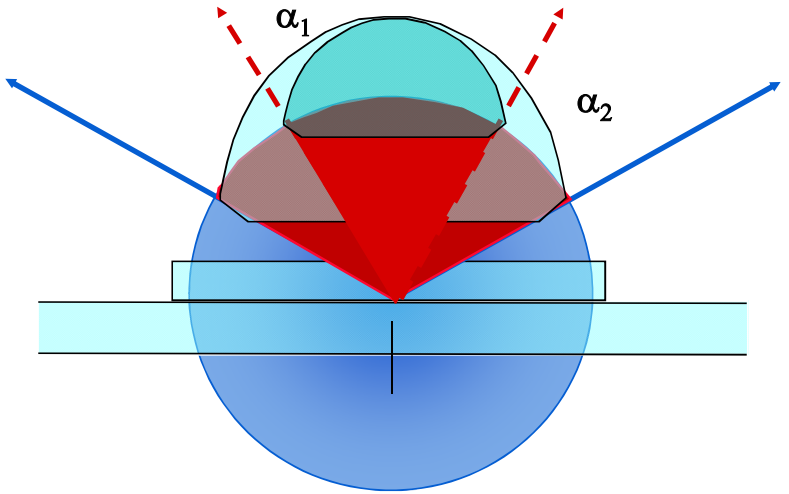
- Depends on the opening angle of the objective and therefore on the NA
- At best it could be 50% since we can only image from one side of the sample (180 degrees)

In reality:

Plan-Apochromat 63/1.4

$$\eta_{NA} = \frac{1}{2}(1 - \cos\alpha) \qquad \eta_{NA} \approx 0.31$$

- Therefore only 31% of the emitted light is collected by the objective



Objective Choice - Light Throughput

inBiotek Microsystems

Light Collection Efficiency

$$10^4 x \frac{NA^2}{M^2}$$

Overall Image Brightness

$$10^4 x \frac{NA^4}{M^2}$$

Magnification	Numerical Aperture	Light Collection Efficiency	Image Brightness
40	**0.65**	**3.5**	**2.0**
40	**1.20**	**9.0**	**13.0**
40	**1.30**	**10.6**	**17.9**
63	**1.40**	**4.9**	**9.7**
100	**1.40**	**2.0**	**3.8**

Use the Highest Possible *NA* and the Lowest Possible *M*

Contrast Techniques

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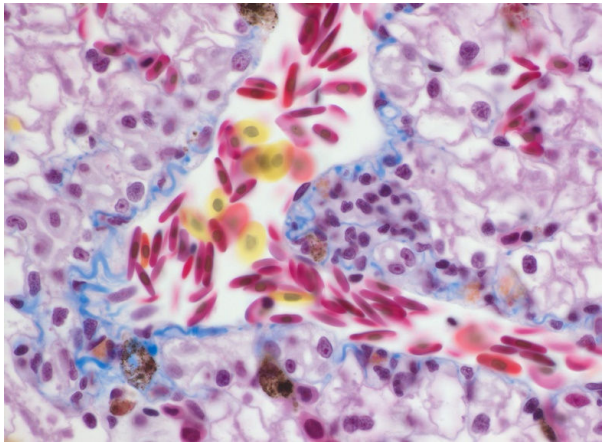
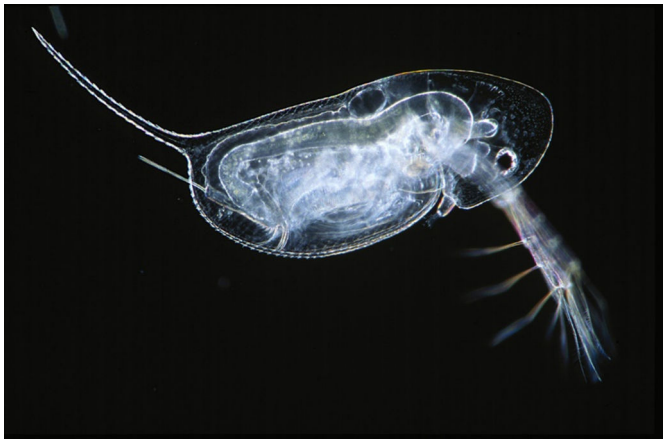
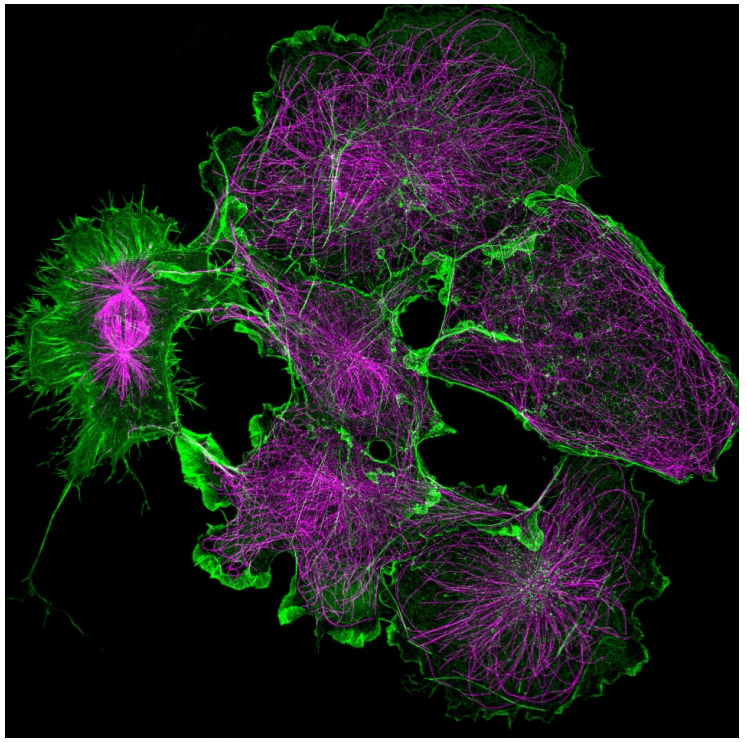


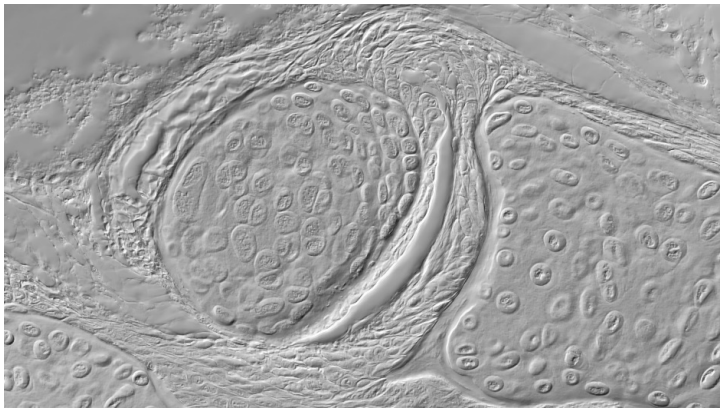
Brightfield



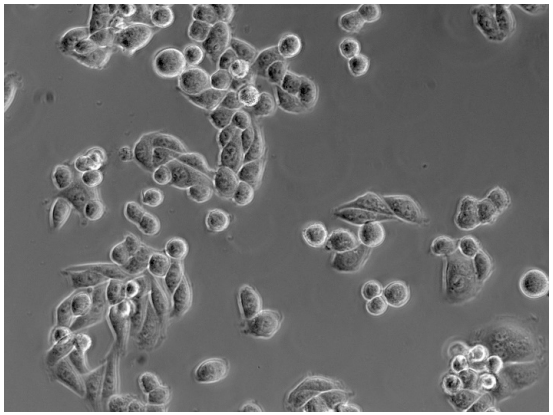
Darkfield



Fluorescent



DIC



Phase Contrast

Contrast in Microscopy

inBiotek Microsystems

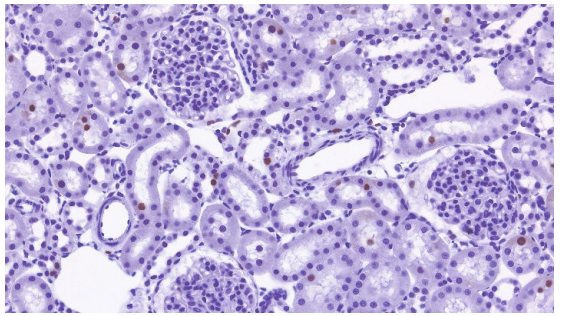
Visibility of a structure against background

- Visibility against background i. e. contrast is necessary to see a magnified and resolved structure of the specimen
- A difference of intensities > 20% is needed between specimen structure (IS) and background (IB) to see contrast.

$$C = \frac{I_S - I_B}{I_S} > 0{,}2$$

Contrast in microscopic specimen

- color of the specimen
- staining of specimen with histologic dyes
- optical contrasting methods to contrast “invisible” specimen



TL Contrast techniques

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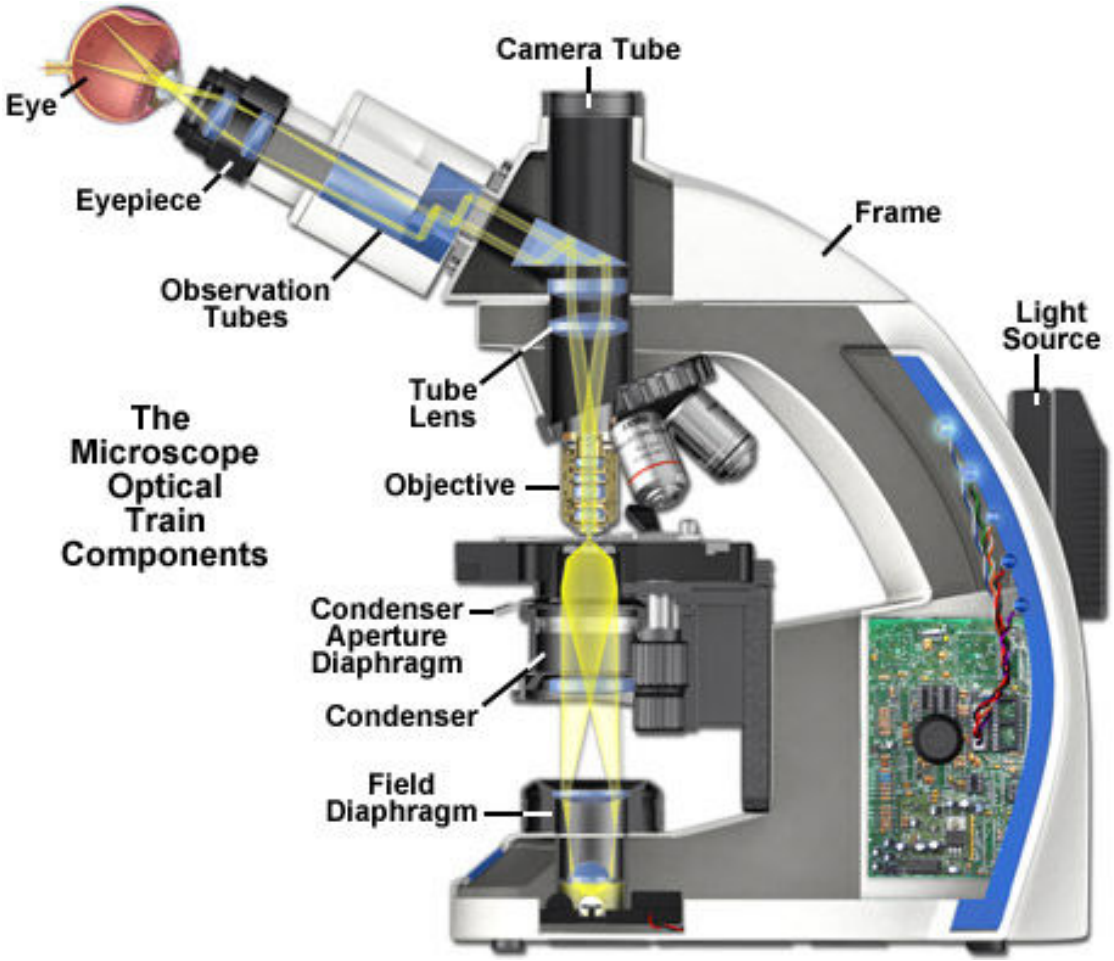
- ❑ Brightfield
- ❑ Phase contrast
- ❑ DIC : Differential interference contrast
- ❑ Darkfield



RL contrast (Fluorescence)

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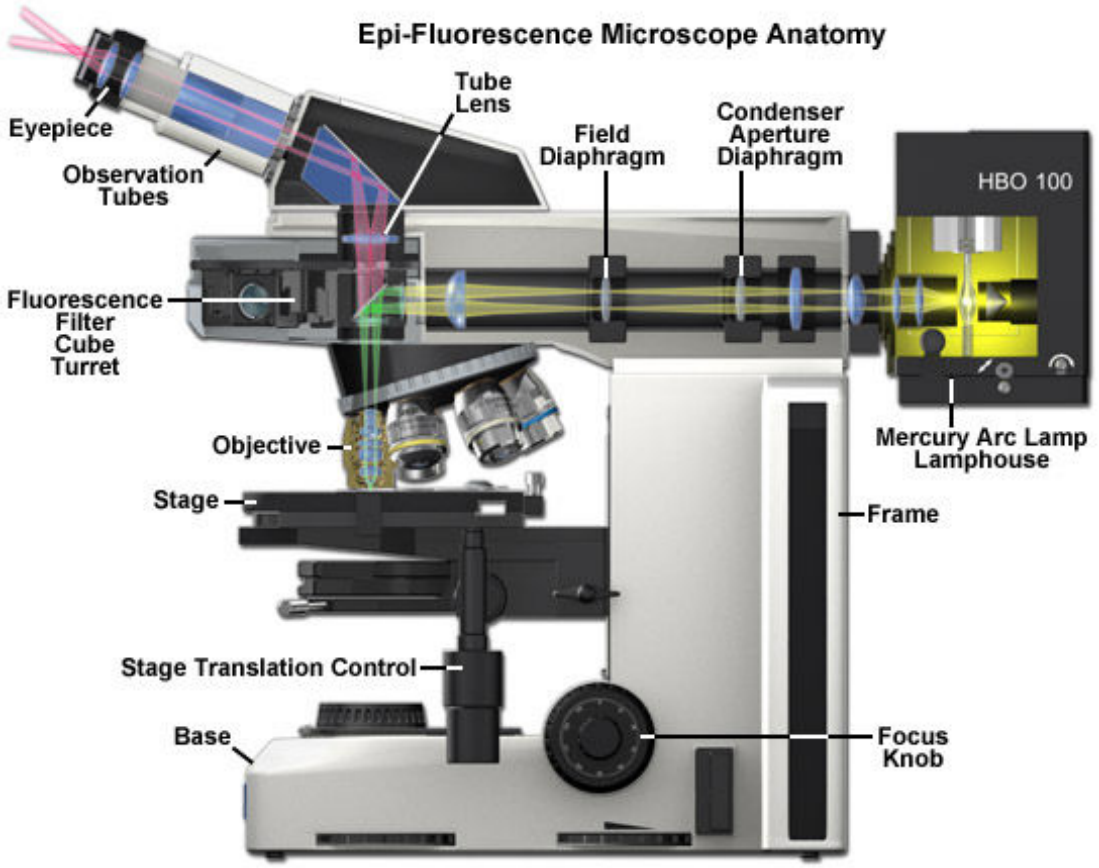


Brightfield TL

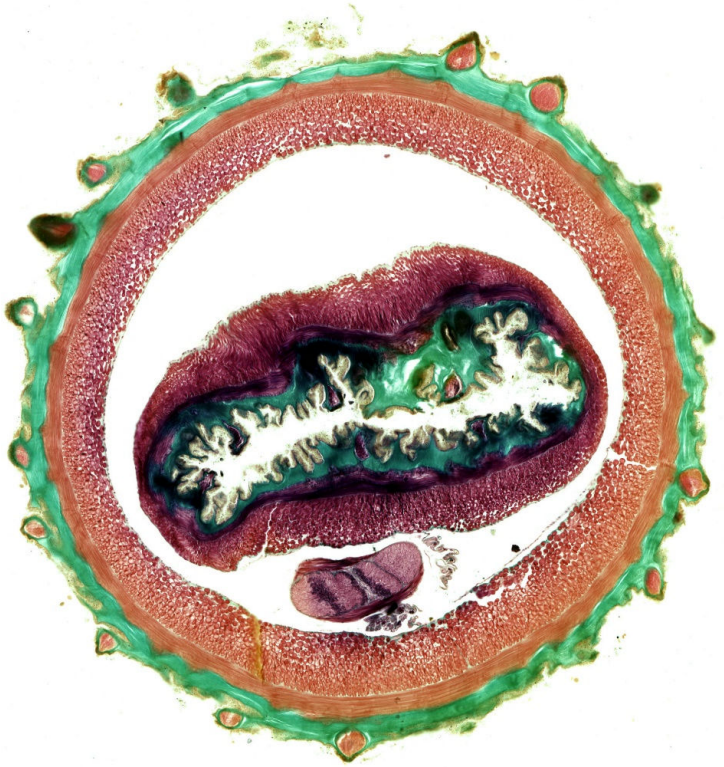
-

- One of the oldest contrast technique
- Most commonly used
- Very simple set up and easy to operate

- colored/stained structures of specimen
- against bright background

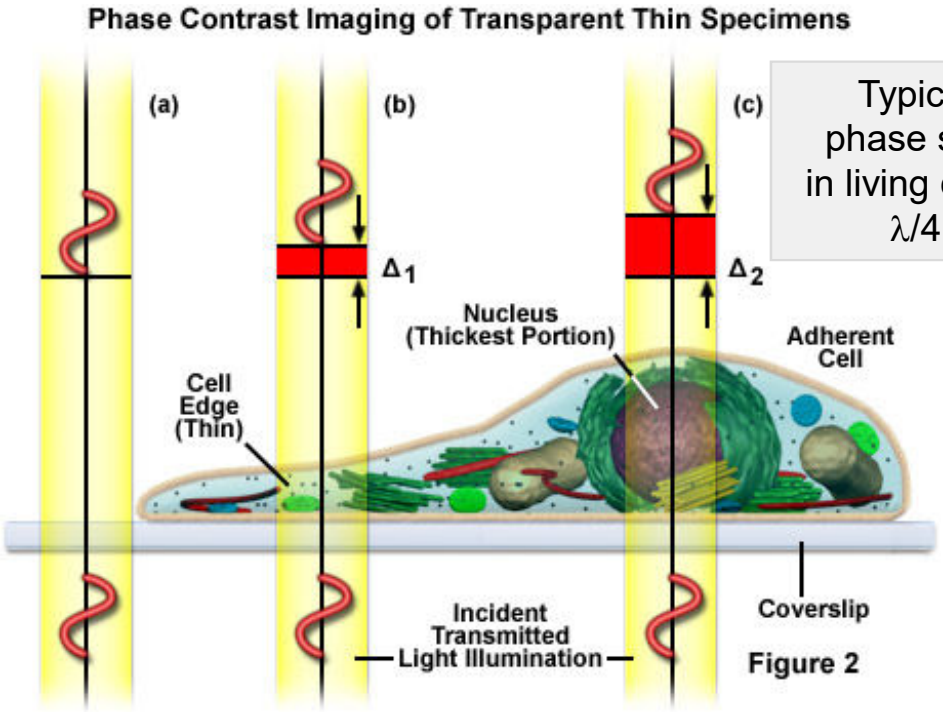
Specimen:
amplitude objects transparent or stained specimen

Design of the microscope:
- classical beam path



Phase Contrast

- Dr. Frits Zernike, The Netherlands1934 development of method, **1953 Nobel Prize**
- Phase objects, mainly unstained living cells in cell culture
- Structures of cells having a slightly higher refractive index than surrounding medium reduce light velocity and diffract light
- The thicker the cellular structure or the higher its optical density the more is the phase shift
- Human eye is not able to see phase shifts



Typical phase shift in living cells: $\lambda/4$

Phase Contrast in Principle

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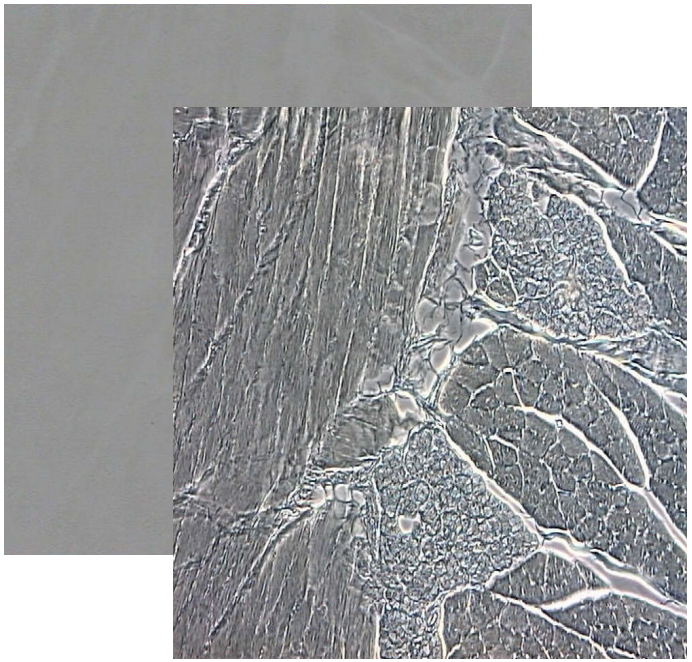
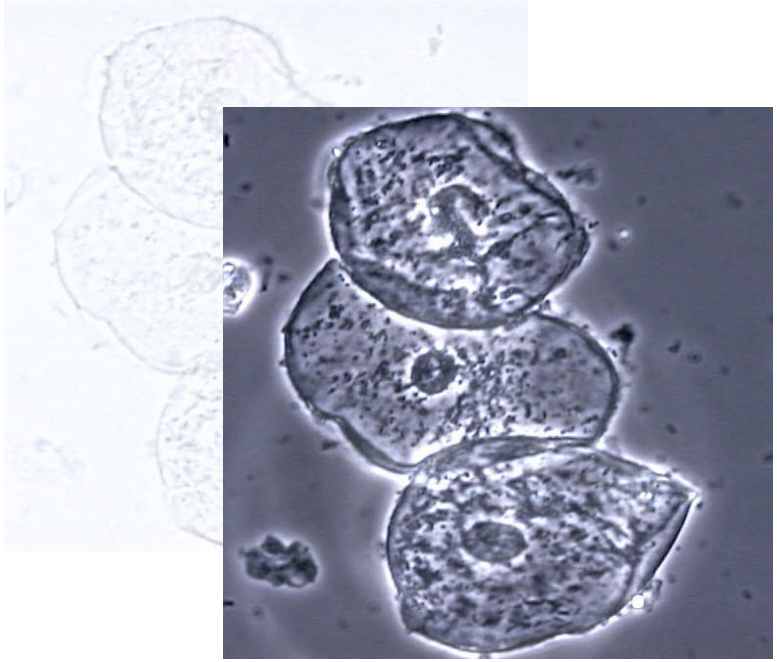
Working principle of phase contrast: Translation of invisible phase shifts into visible differences of intensities





- Result: Specimen structures appear dark against grey background

Phase Contrast

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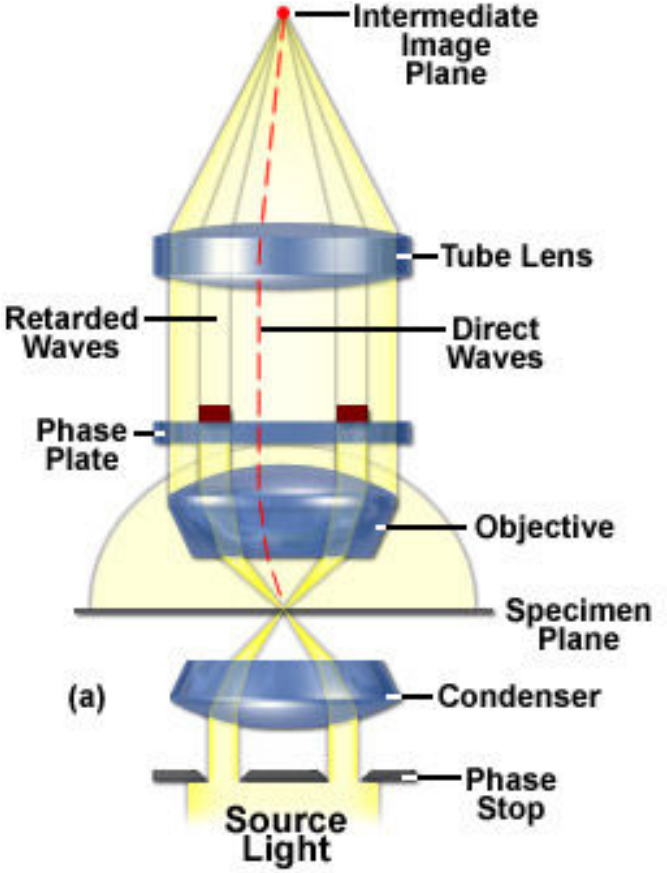
Translates phase differences into intensity differences for fine tissue and cell structures in thin, unstained specimens.

- ❑ Multi-well plate
- ❑ Flask
- ❑ Petri dish
- ❑ Works on both plastic and glass dishes



Phase Contrast - components

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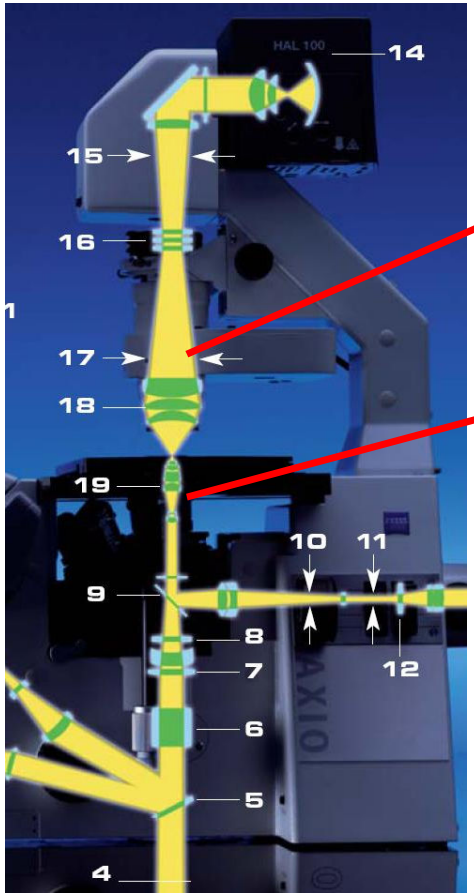
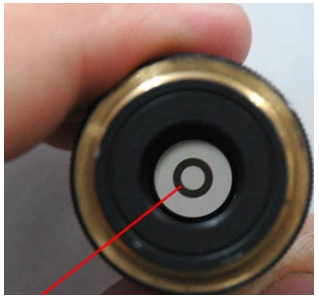
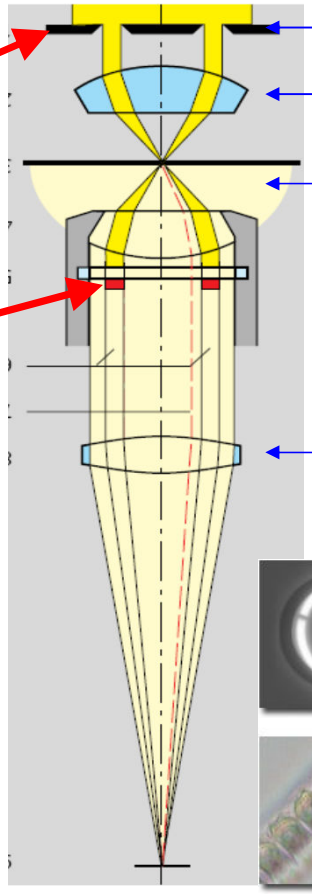


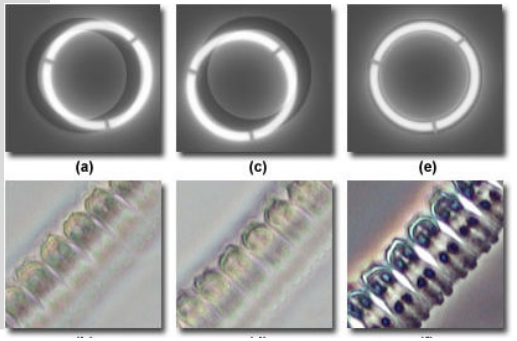
Phase Stop

Phase Ring





Alignment



Differential Interference Contrast (DIC)

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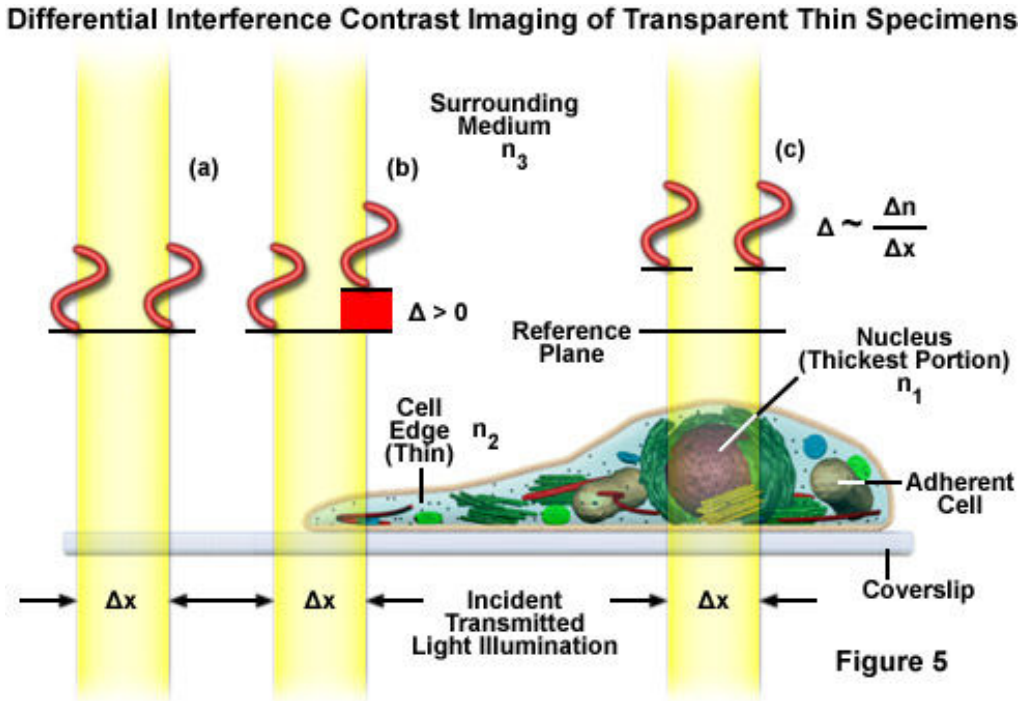
- Splitting of a light beam into two narrow light beams which vibrate in phase
- Both light beams pass independently through specimen. Comparison of the rays after convergence
- If both beams pass through structures with similar refractive indices n:
  - No contrast (A, C)
- If both beams pass through structures with different refractive indices n:
  Pathdifference between the light beams (B)
  Change of light intensity through interference
  Path difference translated in contrast which can be seen in the intermediate image



DIC in Transmitted Light Design

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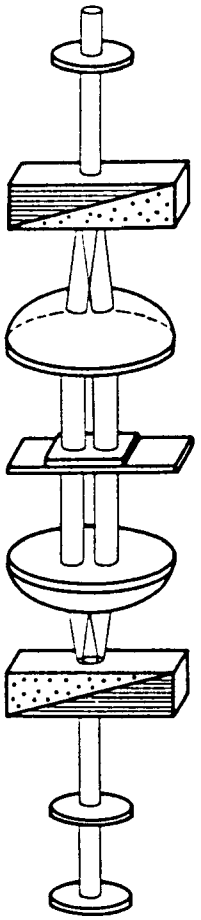
- Polarizer: linearly polarised light
- Wollaston prism in front focal plane of condenser:splits incident light beam into 2 partial beams
- Specimen
- Nomarski prism in back focal plane of objective: Superposes the partial beams
- Analyzer: Selects the common components of the polarized light of the superposed partial beams for interference
- Polarizer and analyzer are crossed polarizing elements



DIC (differential interference contrast)

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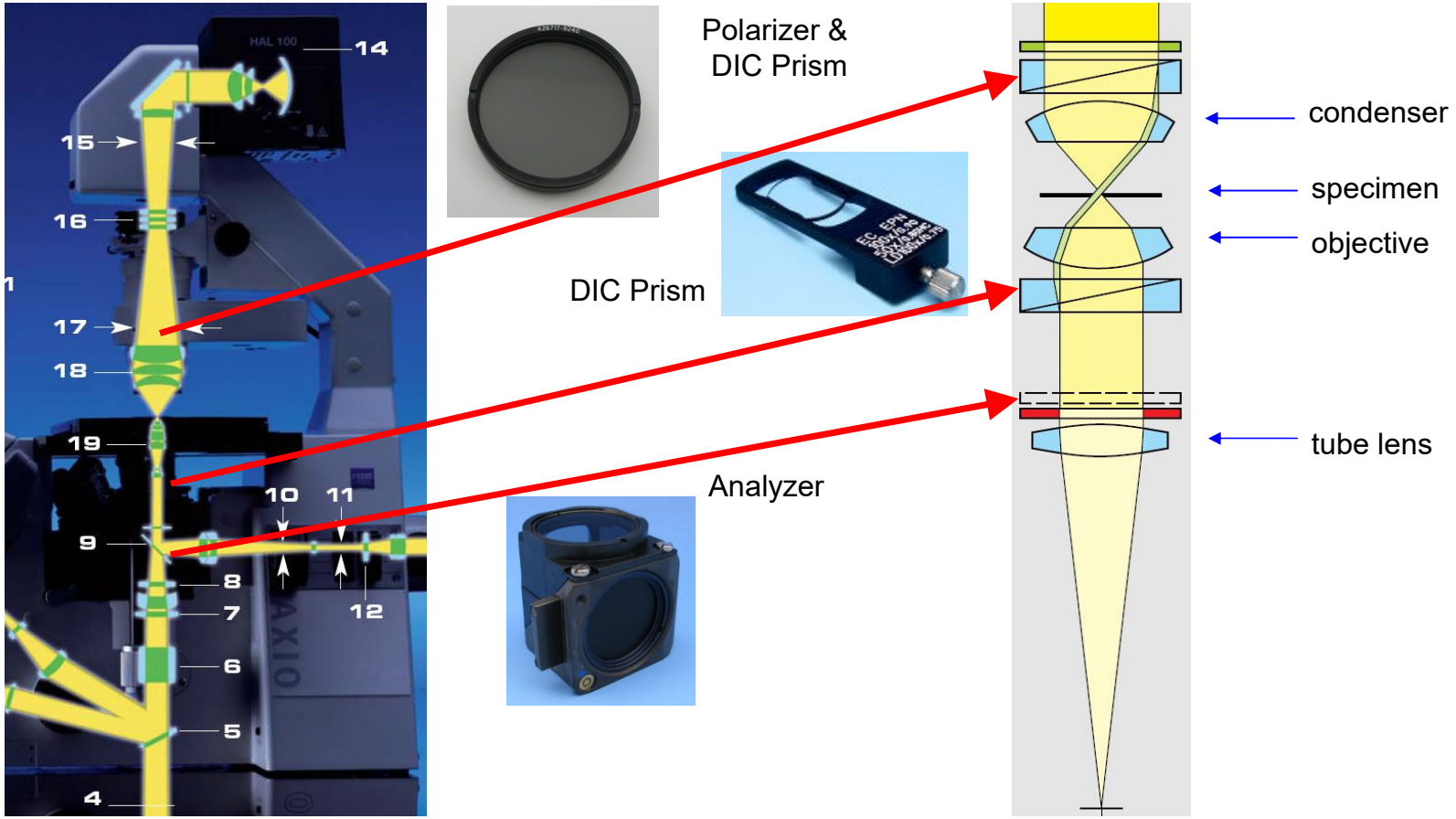
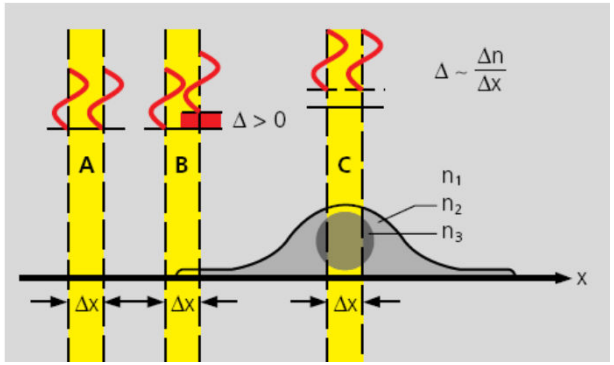


DIC (differential interference contrast)

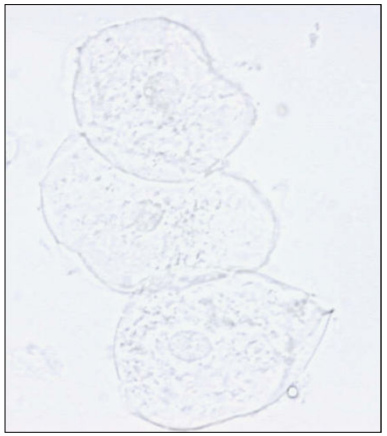
Translates refractive index differences into intensity differences

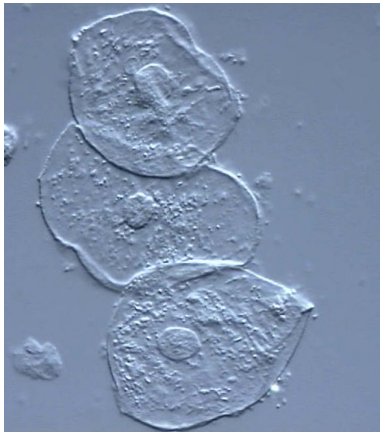
- For thick, unstained specimens
- Require birefringent material
- Does not work on plastic



Brightfield

DIC





Darkfield

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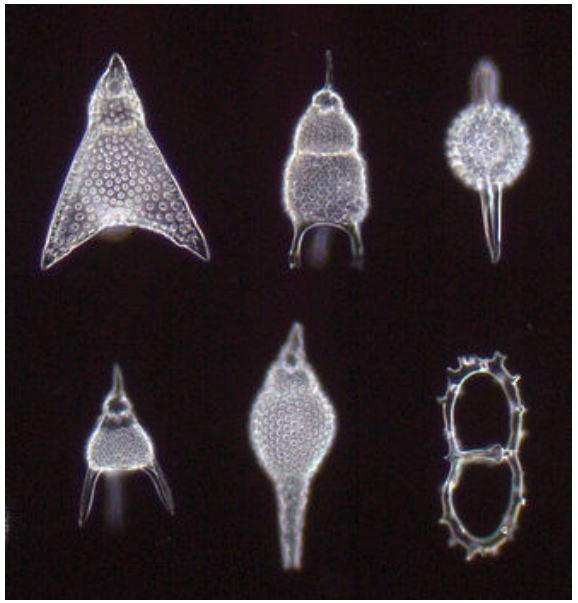
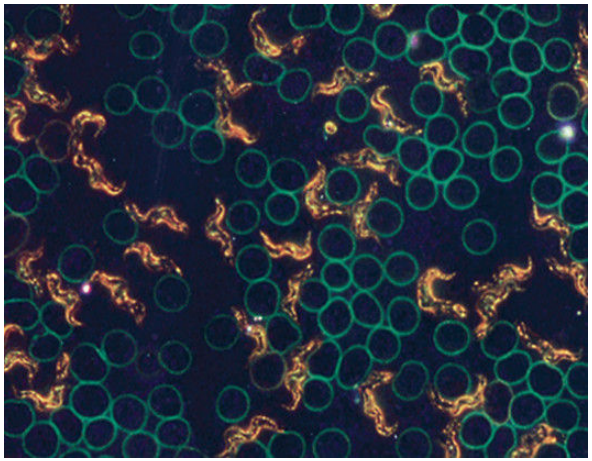
- Bright structures of the specimen against dark background
- Works ideally on unstained sample but can also work with stained samples
- Un-diffracted light does not meet/enter the objective
  - Provides dark background
  - Principal maximum not captured by the objective
- Light diffracted and scattered by the specimen hits the objective lens
  - Bright specimen structures
  - Secondary maxima captured by the objective



The **Radiolaria**, are protozoa of diameter 0.1–0.2 mm



Hematology: Blood smear in darkfield

Darkfield in Transmitted Light Design

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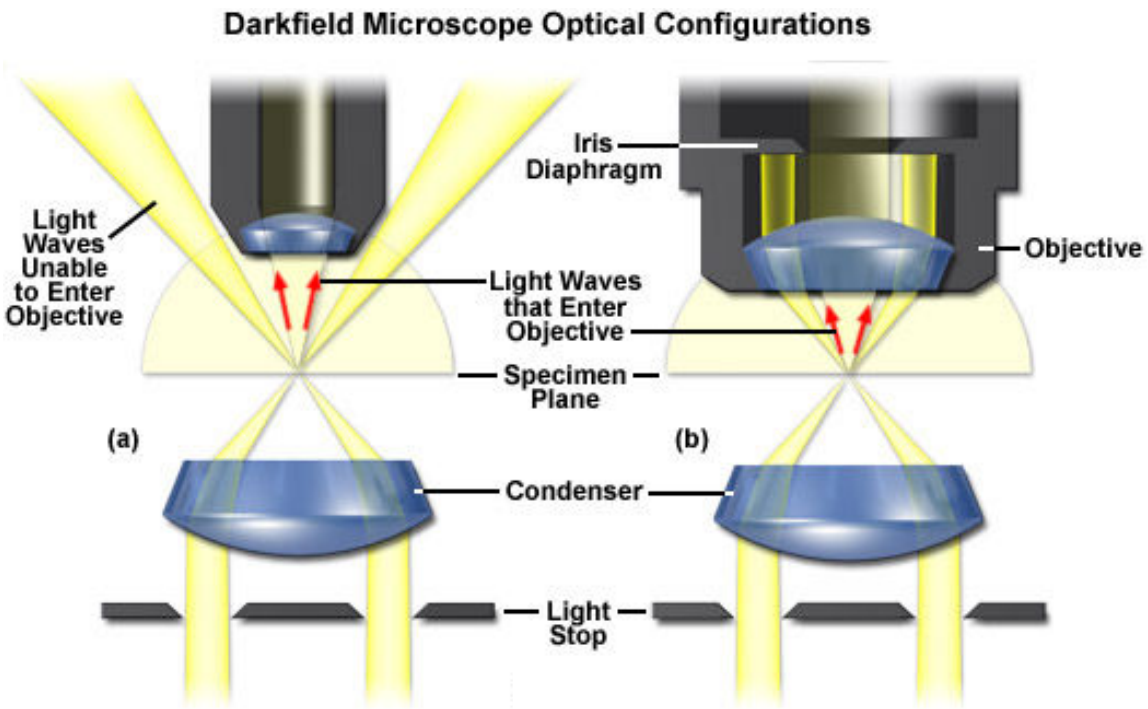
- Specimen
  - cells, bacteria, dust, thrombocytes, structures and particles which are smaller than limiting resolution of the microscope but diffract light
- Design of the microscope
  - condenser with DF ring stop
  - N.A Objective < N.A Condenser
  - Some objectives with high N.A are equipped with a variable aperture iris in the pupil plane to reduce objective aperture for DF observation.



Fluorescence – reflected light path

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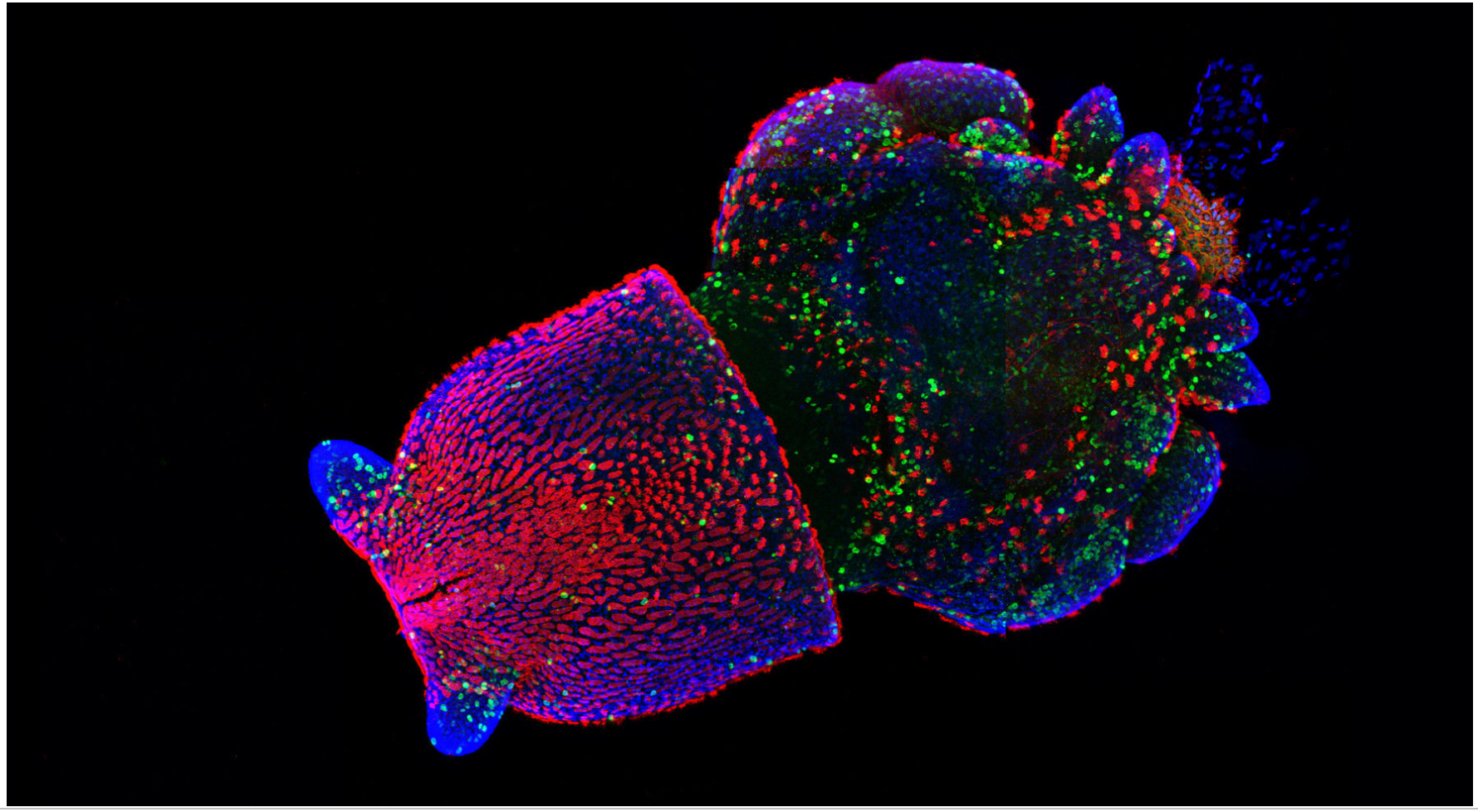

Definition of Fluorescence

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= luminous phenomenon of a substance after illumination

- excitation and emission of light (almost) simultaneously, afterglow < 10^{-6} s after end of excitation
- excitation wave lengths shorter than emission wave lengths

Fluorescence

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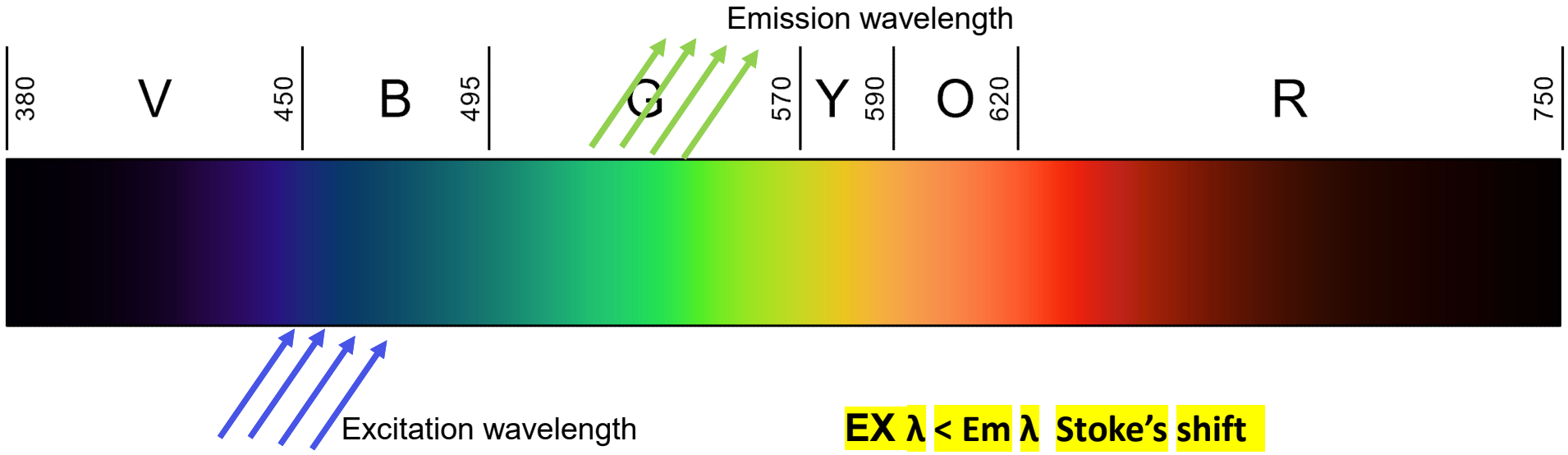
Fluorescence is the property of a substance to absorb light of a particular wavelength and emit light of higher wavelength



EX λ < Em λ Stoke's shift

Blue before green

By Gringer - Own work, Public Domain, https://commons.wikimedia.org/w/index.php?curid=4639774

Fluorescence Microscopy

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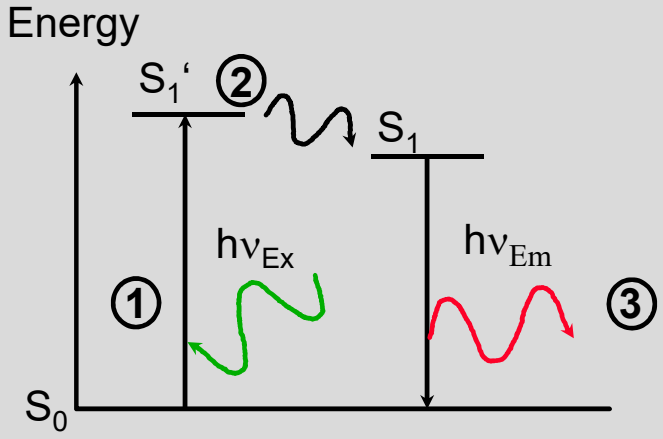
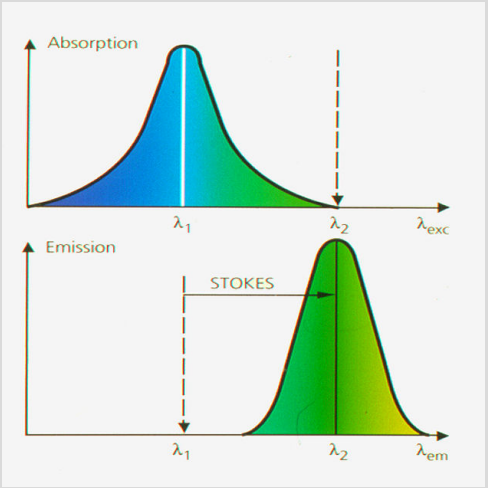
- Light ($h\nu_{Ex}$) excites a fluorophore (1)
- Excitation: energy content of light lifts electrons to a higher level of energy (excited state S_1')
- Partially dissipation of energy (2): lower level of energy (relaxed excited state S_1)
- Emission: Return (3) of electron to ground state (S_0) after < 10^{-6} s. Energy is emitted as fluorescence light of lower enregy ($h\nu_{Em}$)
- Stokes shift = $h\nu_{Ex} > h\nu_{Em}$





Filter set – components and function

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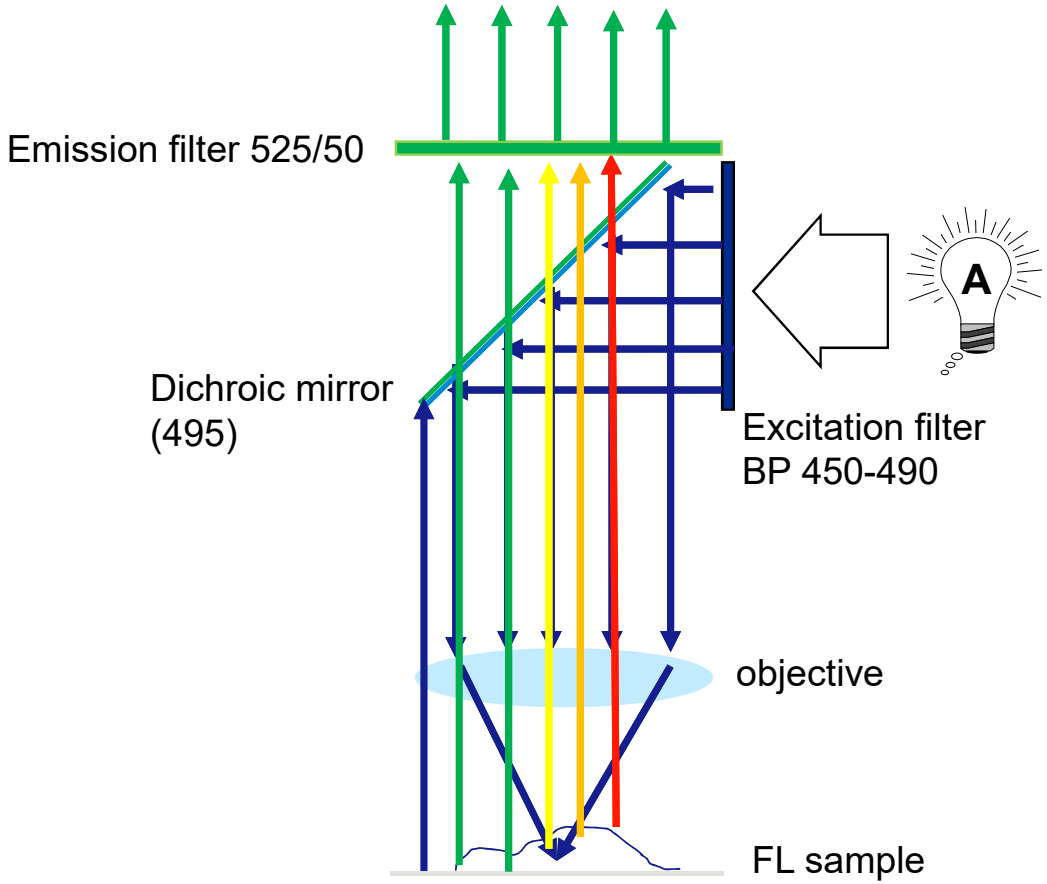
Excitation filter
dichroic mirror
emission filter

FL FILTER SET



Filter sets

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- Band pass : From a particular wavelength till a particular wavelength e.g. BP 450- 490
- Short pass : Anything less than a particular wavelength
- Long pass : Anything above a particular wavelength
- Narrow pass: a specific wavelength only

FL beam path in a microscope

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Components for fluorescence:

- ❑ High intensity white light source
- ❑ Reflector turret to mount filter set/cubes
- ❑ Specific set of filter sets
- ❑ High N.A fluorescence grade objectives
- ❑ FL sample
- ❑ Mono camera

inBiotek Microsystems

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