MICRO-562

Biomicroscopy II

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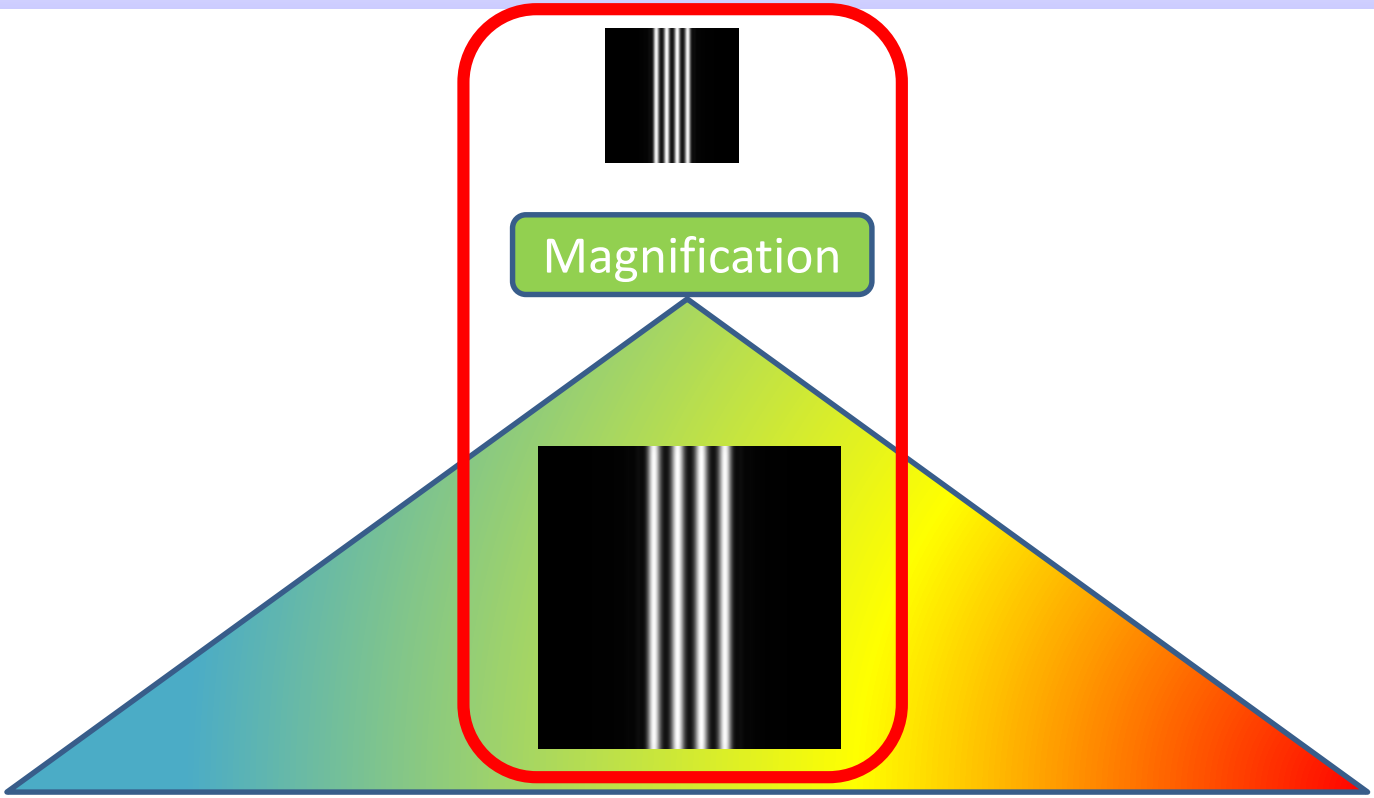
Biomicroscopy-II Syllabus – (TENTATIVE)

Lecture 1	Course	Brief Review, Dark-Field
Lecture 2	Course	Phase-Contrast, Polarization, Birefringence
Lecture 3	Course	DIC, Fluorescence Microscopy Techniques
Lecture 4	Course	Introduction to Confocal Microscopy
Lecture 5	Course	Advanced Microscopy-I
Lecture 6	Course	Advanced Microscopy-II
Lecture 7	TP	Practical: Intro to Wide-Field Microscopy
Lecture 8	TP	Practical: Transmission Techniques
Lecture 9	TP	Practical: Intro to Fluorescence
Lecture X	**EASTER**	
Lecture 10	TP	Practical: Intro to Confocal
Lecture 11	TP	Practical: Fluo/Conf
Lecture 12	TP	Practical: Fluo/Conf
Lecture 13	**EXAM**	
Lecture X	**HOLIDAY**	

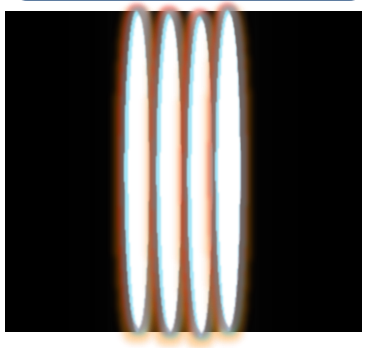
Optical Microscopy

- Relatively **non-invasive**
- **Sensitive**, it can follow distributions and interactions **down to the molecular level**
- Can be **quantitative**
- Can be applied to **live cells** to monitor biological events with temporal and/or spatial resolution
- Allows **external manipulation (i.e. physical/electrical/fluidic probes)**

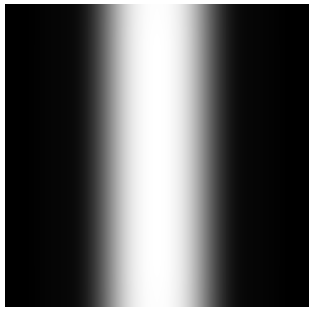
Review: Important aspects for microscopy



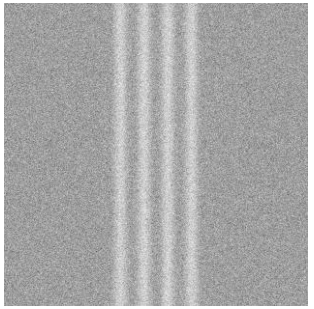
Geometrical aberrations



Resolution

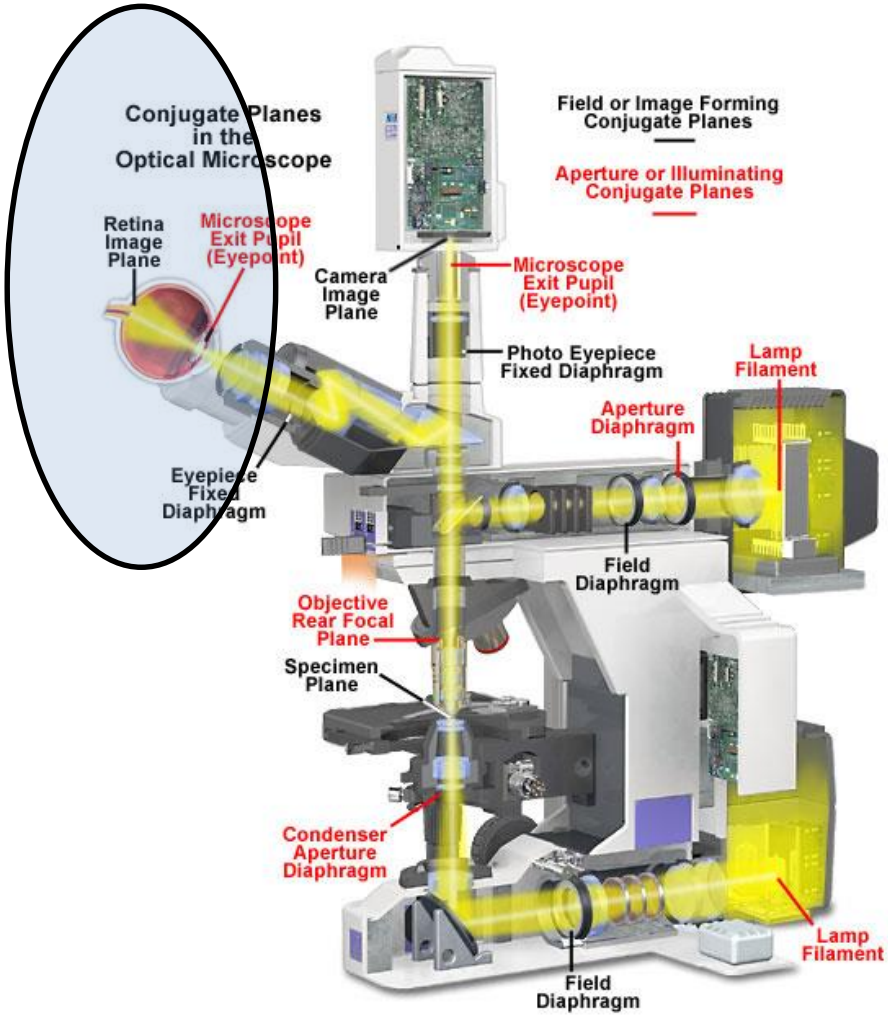


Contrast



Review: Microscope – An Optical System

→ Optics is used to generate a larger image on the **retina**

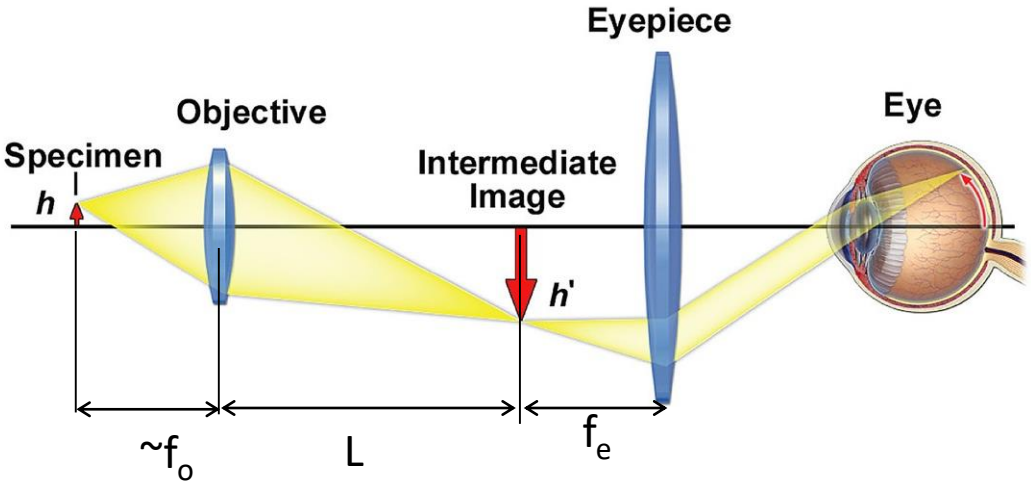


Review: Compound Microscope

Objective lens followed by **an eyepiece** (a.k.a ocular):

Objective: forms real & magnified image (at a plane where the instrument's field stop is located)

Eyepiece: Its object plane is the objective's image plane → forms a virtual image at infinity
→ magnified image can be viewed with relax (unaccommodated) eye.



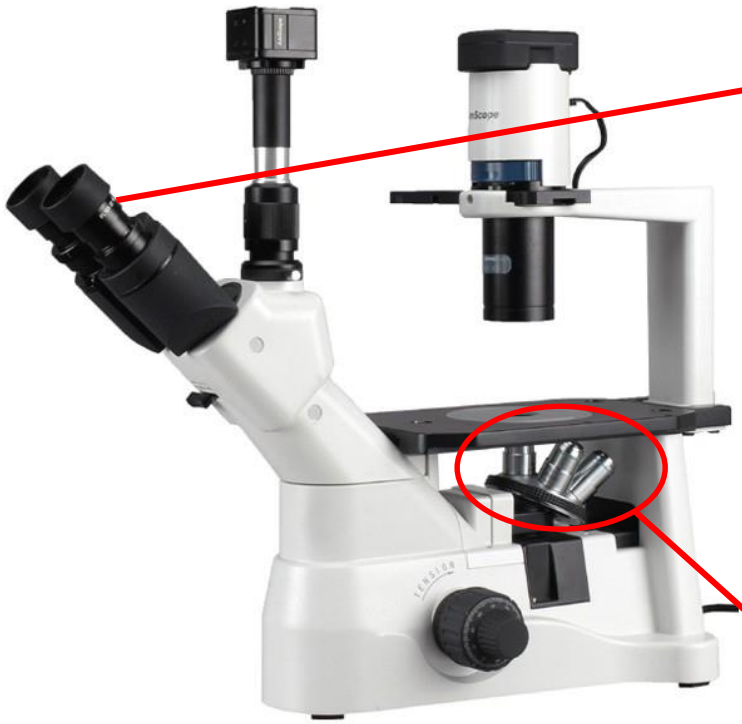
Compound magnifying power is the product of the magnifications of the two elements:

$$MP = M_T^{objective} \times M_A^{eye\ piece}$$

Magnification can reach:
~10 X 100 → ~1000

Review: Optical Microscopes - Magnification

Inverted Microscope



eyepiece

An example eyepiece: 10x

Upright Microscope

objective

An example objective set:
4x, 10x, 20x, 100x

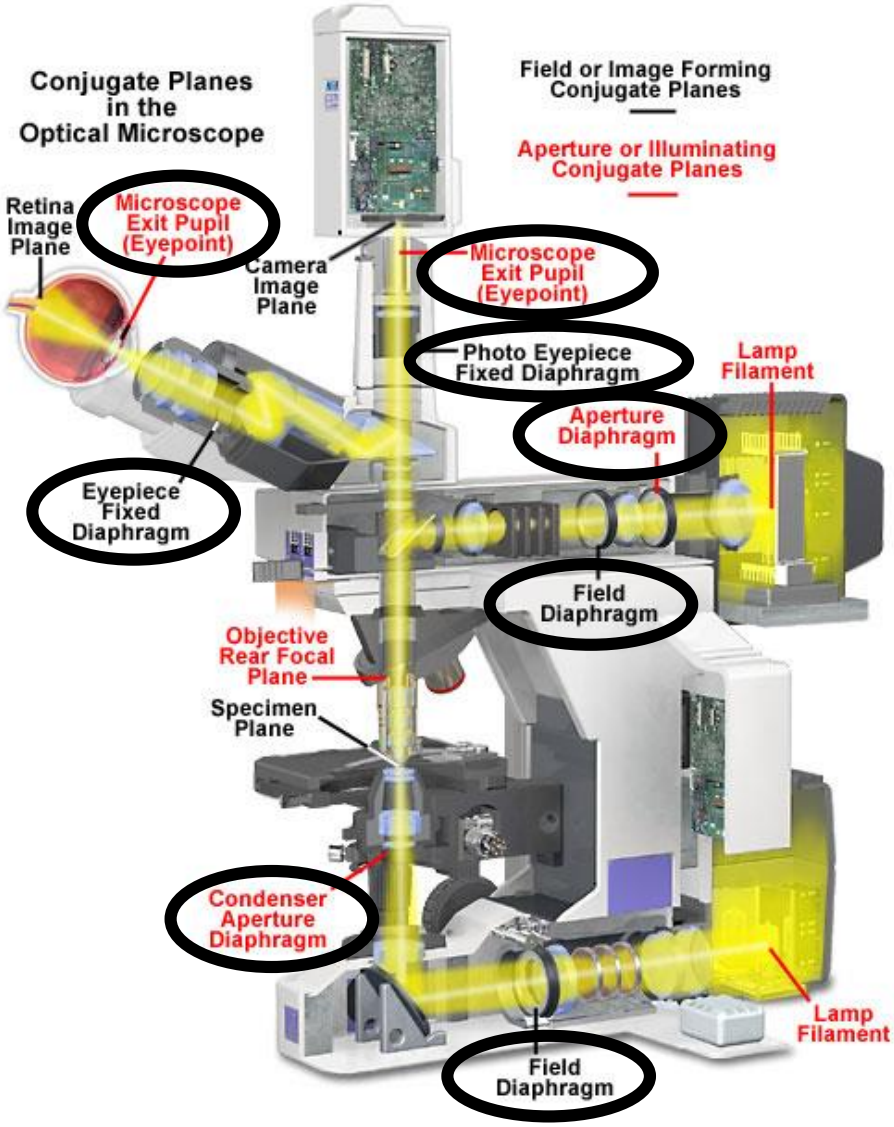
Review: Important aspects in microscopy:

- Magnification
- **Image quality – aberrations, alignment, illumination condition etc**
- Resolution
- Contrast

Major Optical Aberrations in Microscopy

- The major six aberrations are:
    - Spherical aberration
    - Chromatic aberration
    - Coma
    - Astigmatism
    - Curvature of field
    - Distortion
- Lenses are associated with many of these intrinsic optical faults that can distort the image quality.
- **Aberrations can be corrected by measures** such as using compound lens designs, using glass elements with different refractive indexes and color dispersion, incorporating aspherical lens curvatures …
- **Objectives** are designed to correct for aberrations, but they can never completely remove them.
- It is common that a solution for correcting one fault worsens other faults, so the user must prioritize the goals for optical performance and then work toward the best compromise in correcting other aberrations depending on the sample/application. For these reasons, objectives vary considerably in their design, optical performance, and cost.

Review: Stops in Optical System



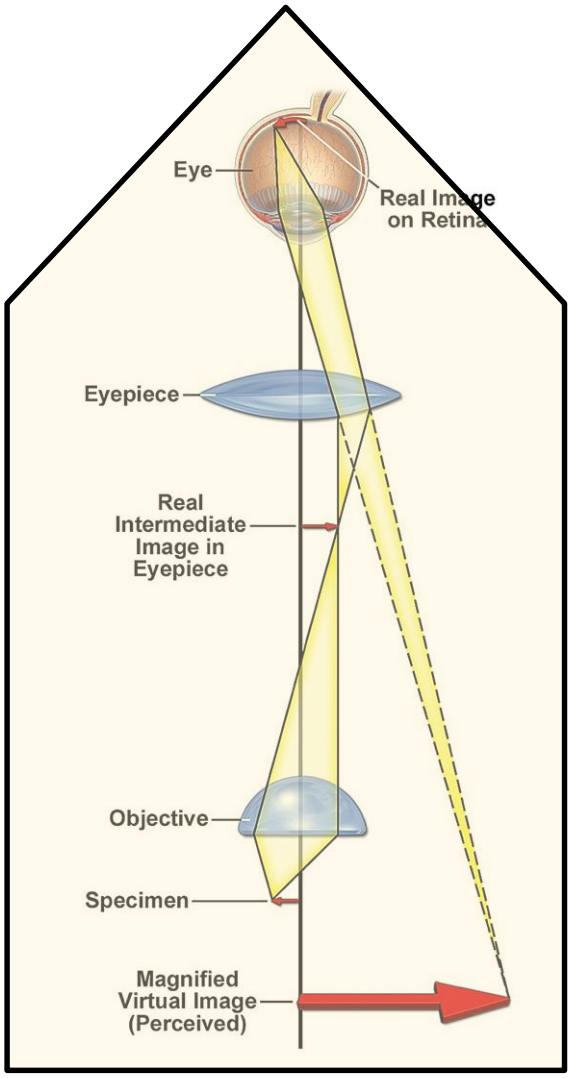
➔lateral limitations act on the overall system performance, i.e. image quality

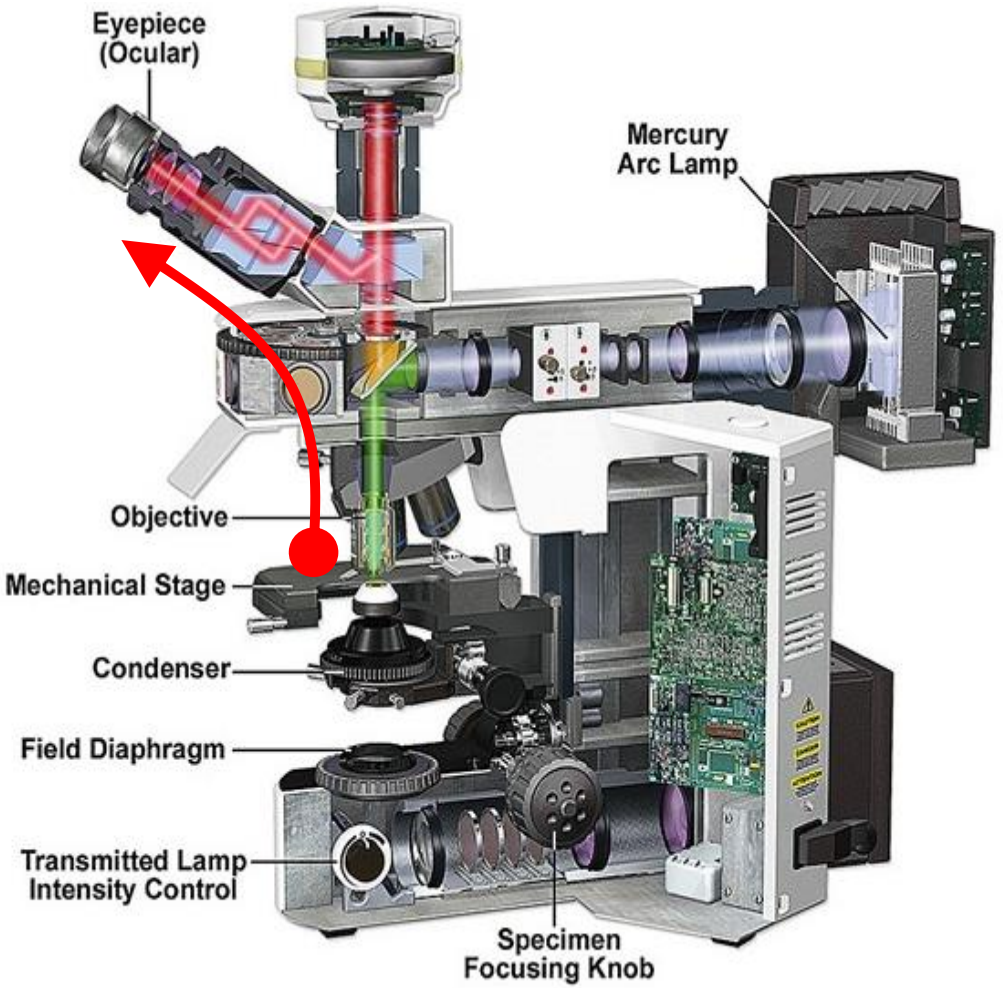
- Aperture Stop/Diaphragm
- Entrance Pupil
- Exit Pupil

- Field Stop/Diaphragm
- Entrance Window
- Exit Window

Review: Imaging path is half of the story….

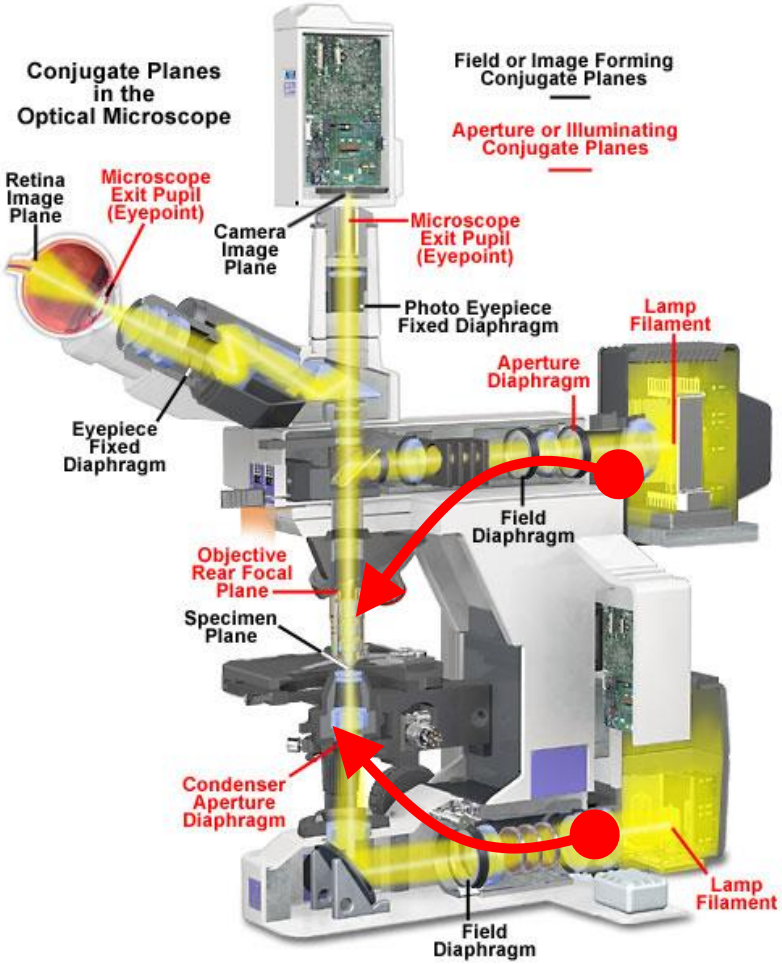
An imaging system with 2 lenses:





Review : …illumination path is the other half

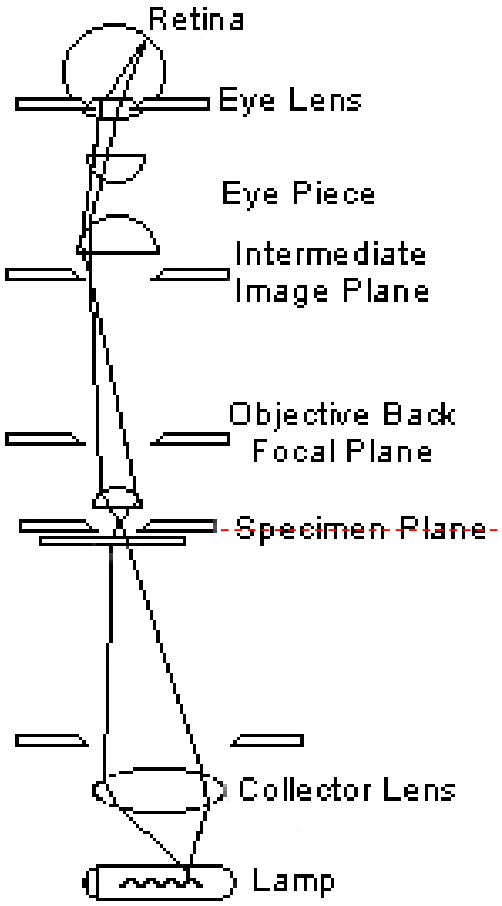
Illumination contributes to the performance of the microscope.



Research grade light microscope with upright stand:
Two lamps provide transmitted and reflected light illumination.

Review: Illumination Schemes

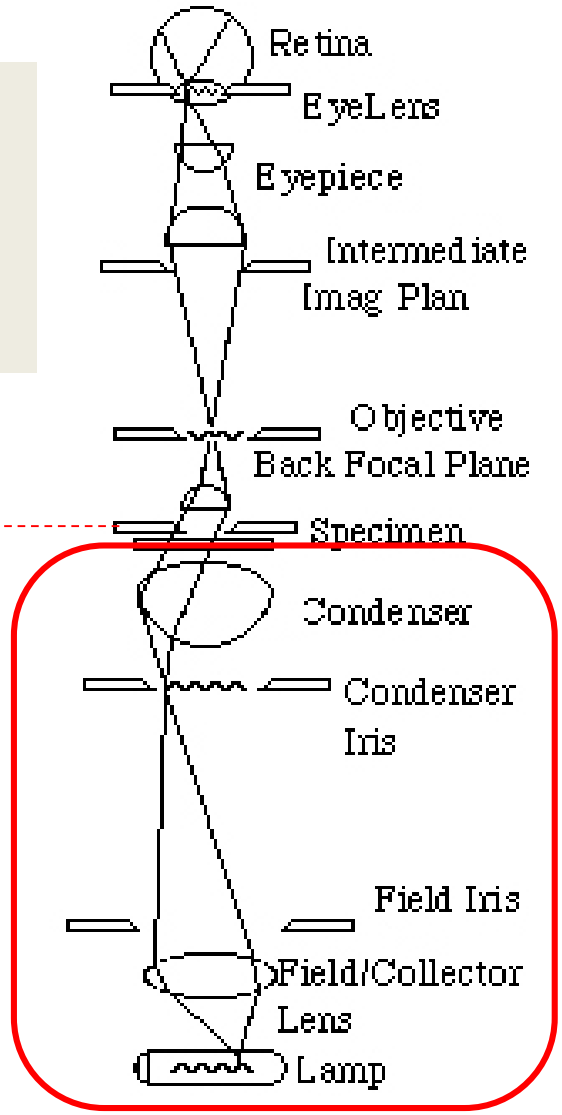
Critical **vs** **Kohler**



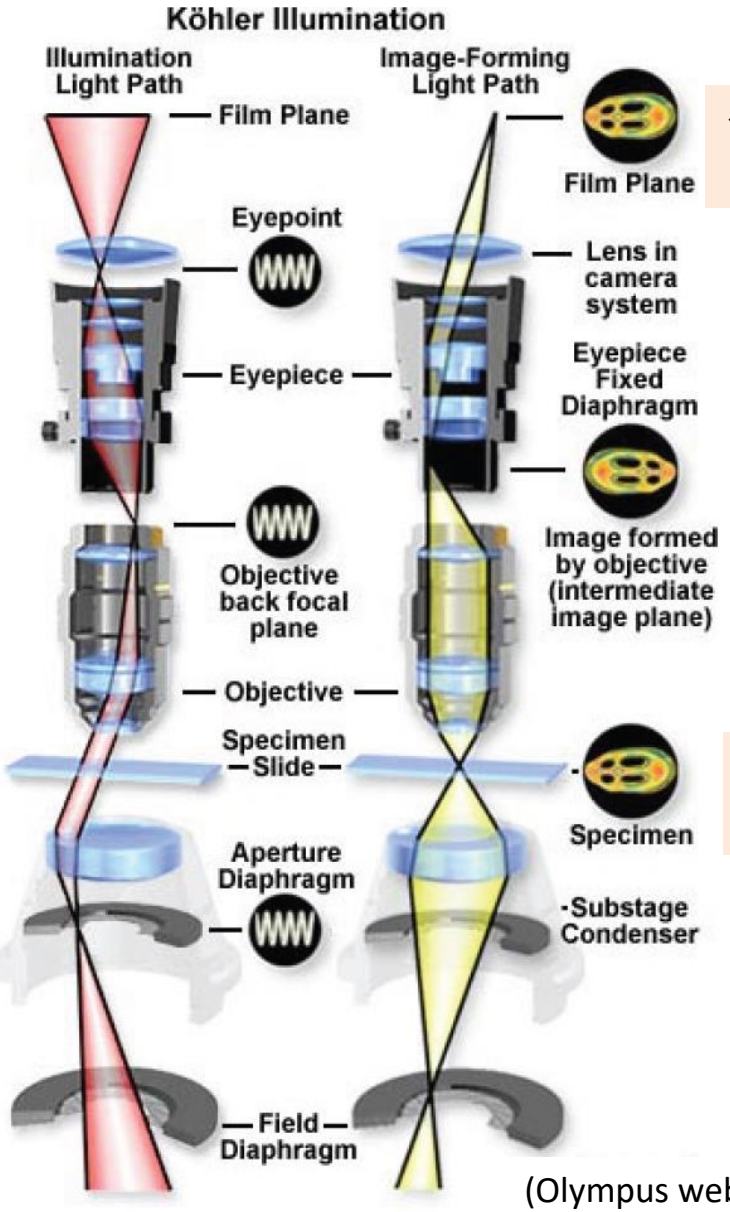
- Critical illumination focuses the lamp directly on the specimen.
- This results in:
  - Uneven & dim illumination
  - Overlap of bulb image with the specimen

Kohler illumination uses **collector-condenser** pair to solve such artifacts:

- Lamp is maximally out-of-focus at the specimen plane
- Provides homogenous illumination
- Improves resolution & visibility
- Minimizes stray light



Review: Bright-Field Kohler Illumination



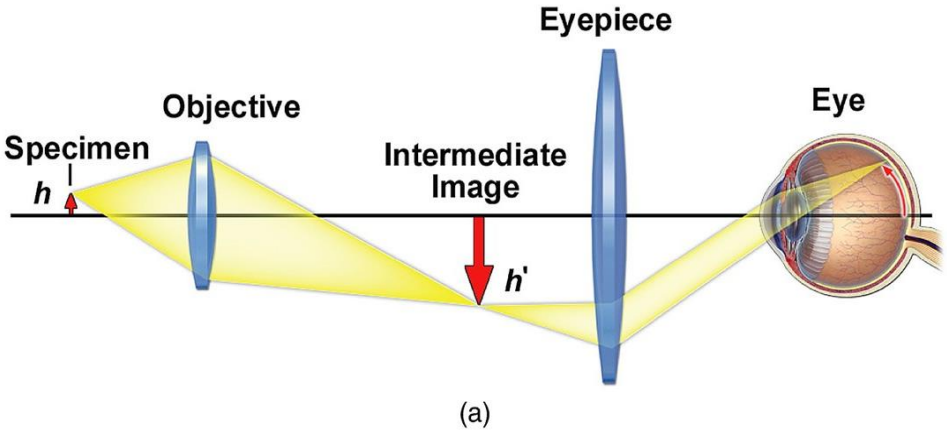
(Olympus web-site)

← Specimen is in-focus at the detector/eye plane

Lamp is maximally out-of-focus ➔ at the specimen plane

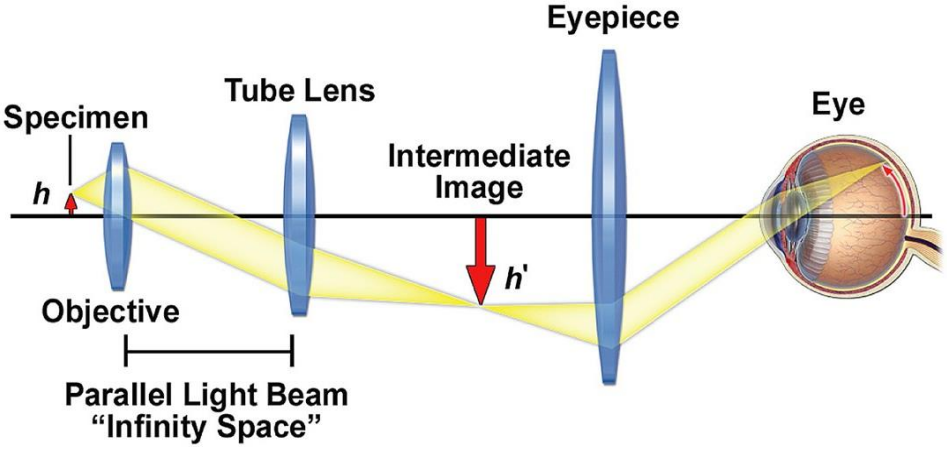
← Specimen is in-focus at the sample stage

Reminder: Infinity Corrected Compound Microscope



(a)

(a) finite corrected microscope configuration



(b)

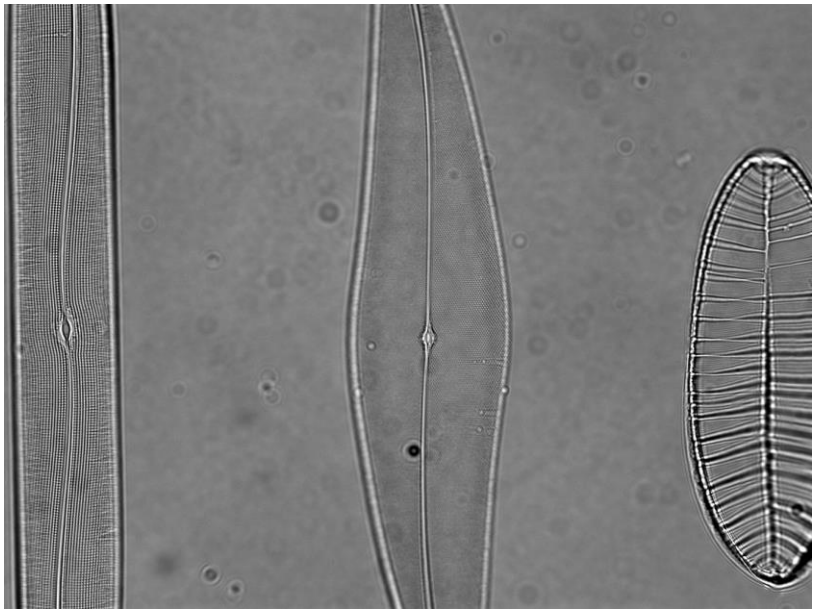
(b) infinite corrected microscope configuration

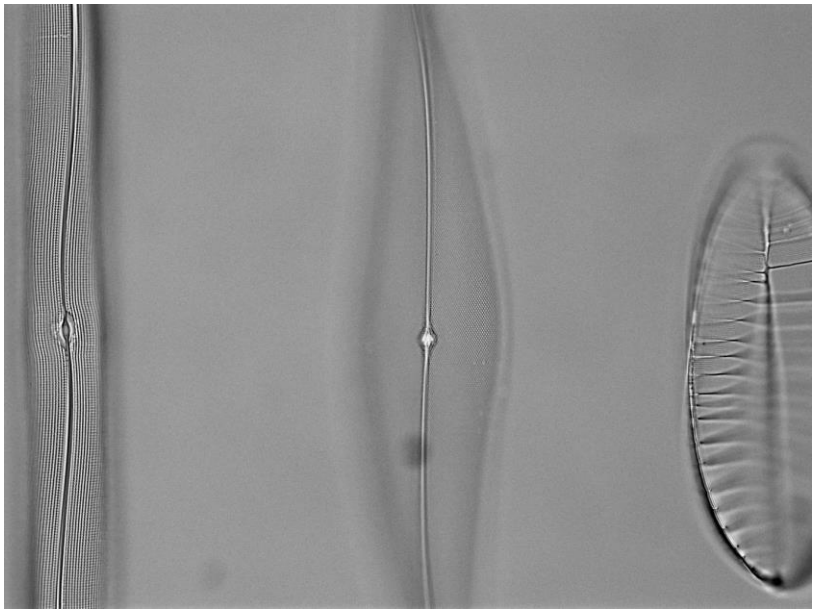
- Infinity optical systems have a different objective design that produces a flux of parallel light wavefronts imaged at infinity, which are then brought into focus at the intermediate image plane by a special optic termed *tube* lens.
- The region between the objective rear aperture and the tube lens is called *infinity space*, where auxiliary components can be introduced into the light path without producing r optical aberrations.

Caution: objectives designed for older 160-mm fixed tube length microscopes are not interchangeable with newer infinity-corrected microscopes.

Review: Image artefacts

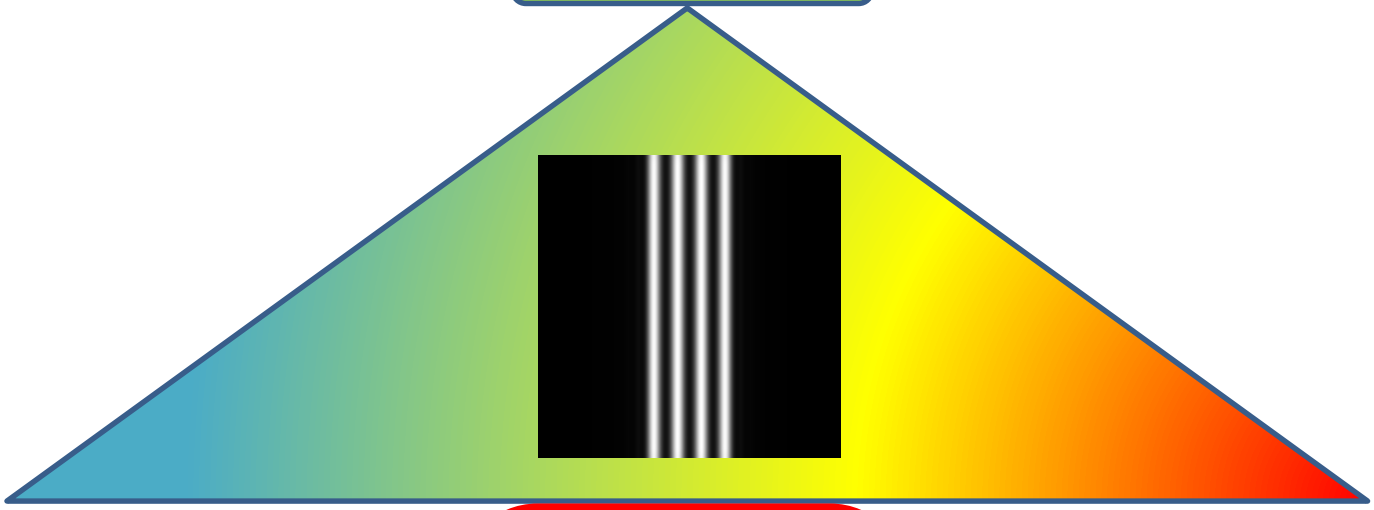
Two images of same object (sample)
imaged with the same microscope (even the same objective) with
different illumination conditions
(aperture stop).



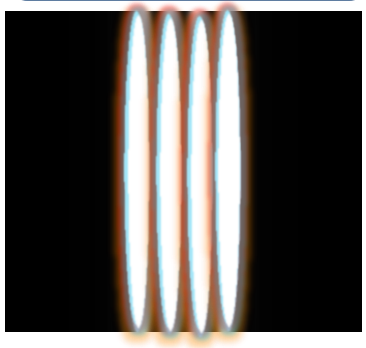


Review: Important aspects for microscopy

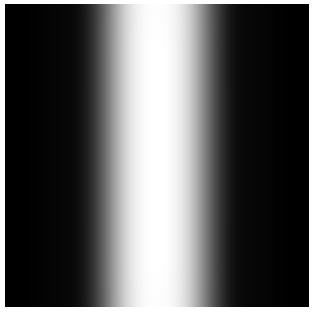
Magnification



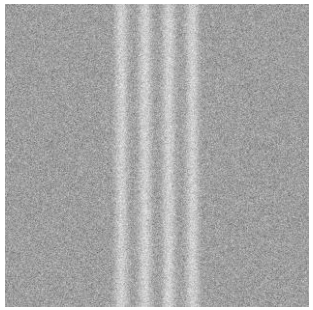
Aberrations



Resolution



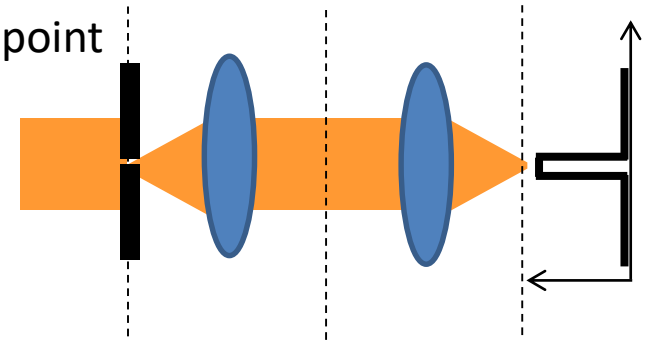
Contrast



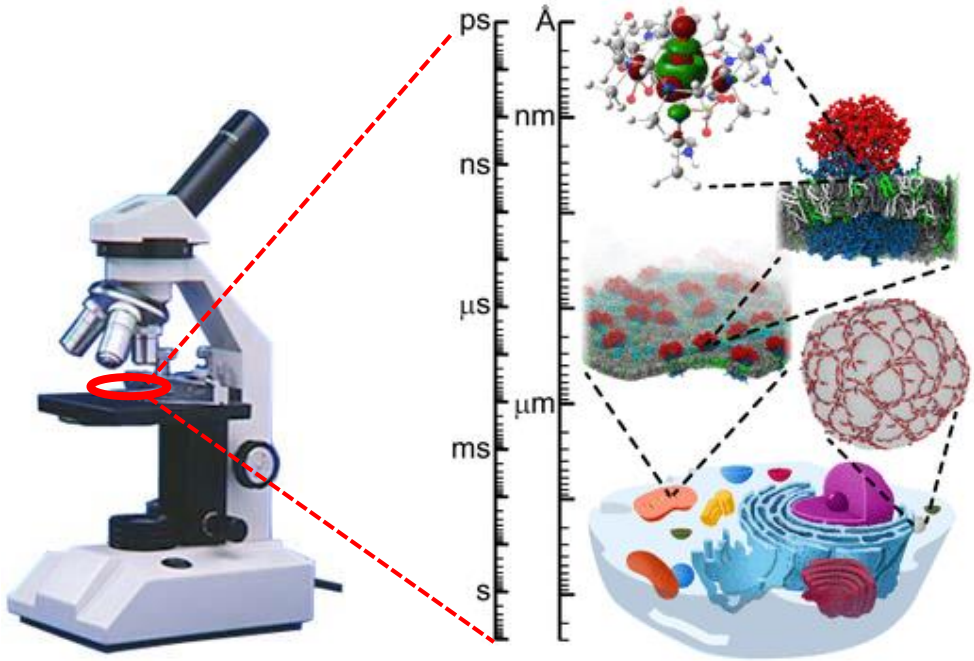
Review: Resolution in microscopy – Ideally

Object/Specimen:
Pin-hole
(represents a point source)

Image:
Ideally, exact copy of the object & magnified.



Ability to image an exact copy implies that a microscope can see **any** object: cell, nucleus, proteins, DNA, atoms …

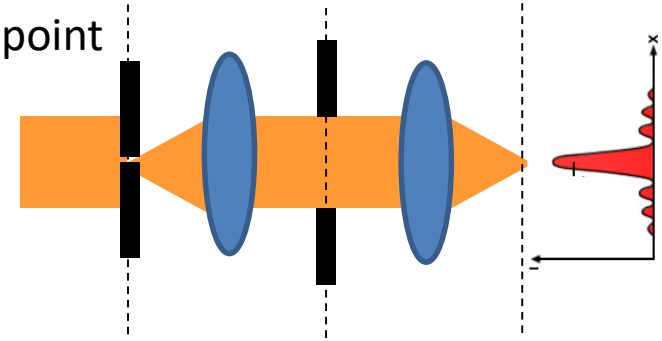


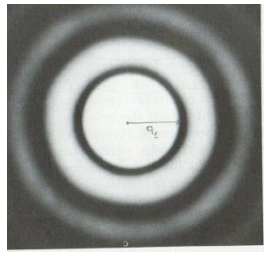
Review: Resolution in microscopy – Reality

Object/Specimen:
Pin-hole
(represents a point source)

F.P
Aperture

Image:
In reality, it is a Point-Spread-Function (PSF)





At object Plane: Put as input an ideal point source (~zero diameter) → $\delta(x)$

At the Fourier plane: "Reality" can be modeled by considering a circular aperture in the F.P.

→Remember that aperture cuts higher frequencies!

→This means "removal" of some information about the object

→ In practice this will result in "blur" images

(here aperture represents aperture stops, finite size of the lens etc

At the image plane: We get the F.T. of the aperture → diffraction pattern

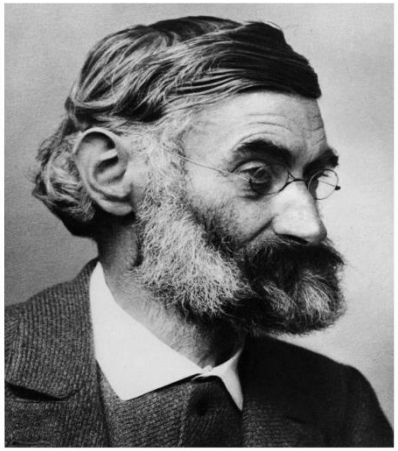
Review: **Microscopy resolution** by 2 methods

1. **Rayleigh criteria**

→ Diffraction of a circular aperture & Airy disks

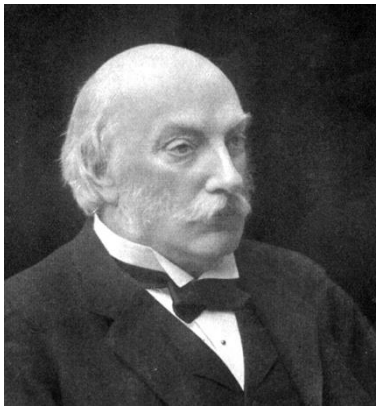
2. **Abbe's theory for image formation**

→ Collecting more **diffraction orders** for higher resolution



Ernst Abbe [1840-1905]

Collaborated with Carl Zeiss (owner of a microscopy company) & Otto Schott (owner of a glass company) in Jena, Germany.



The Lord Rayleigh (**John William Strutt)** [1842-1919]

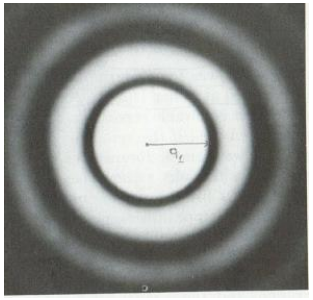
Discovered Rayleigh scattering (which is used to explain why the sky is blue!). Discovered Argon (with Ramsay), got Nobel Prize for Physics in 1904

Review: Determining resolution with 2 methods

1. **Rayleigh criteria**

→ Diffraction of a circular aperture & Airy disk

$$d_{min} \approx 0.61 \frac{\lambda_0}{NA} \approx 0.61 \frac{\lambda_0}{nsin(\theta)}$$



2. **Abbe's theory for image formation**

→Collecting more diffraction orders for higher resolution

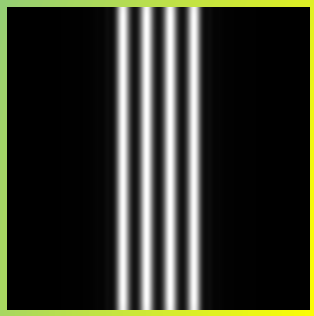
$$d_{min} \approx 0.5 \frac{\lambda_0}{NA} \approx 0.5 \frac{\lambda_0}{nsin(\theta)}$$

Review: Numerical Aperture (NA) & Resolution

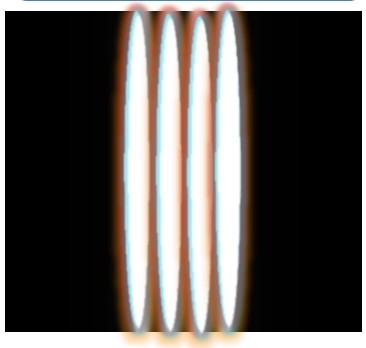
The specimen is an allium (onion) root tip and illustrates a phases of mitosis (you are looking at the chromosomes splitting).

Review: Important aspects for microscopy

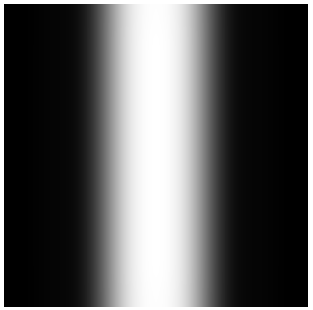
Magnification



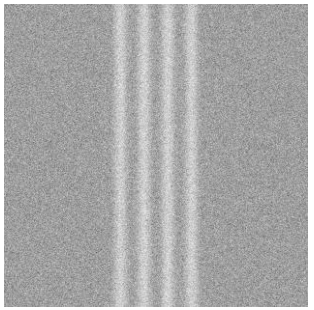
Aberrations



Resolution



Contrast



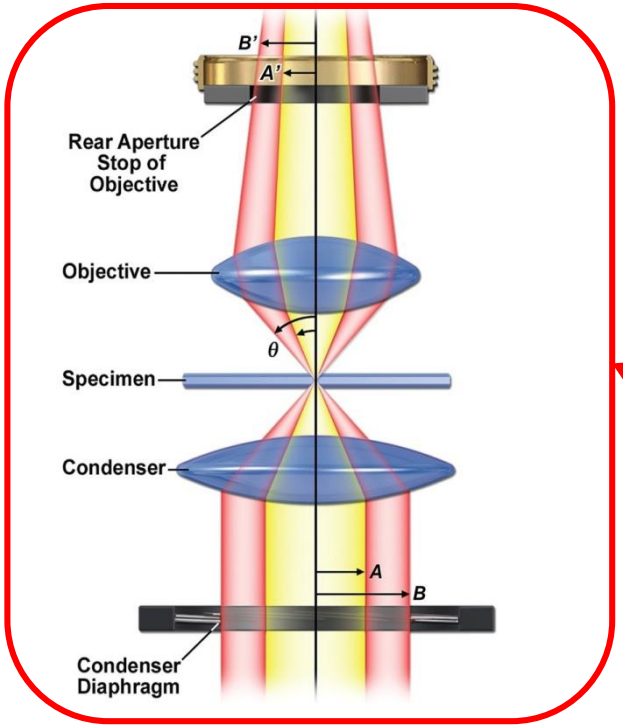
Review: Important aspects in microscopy:

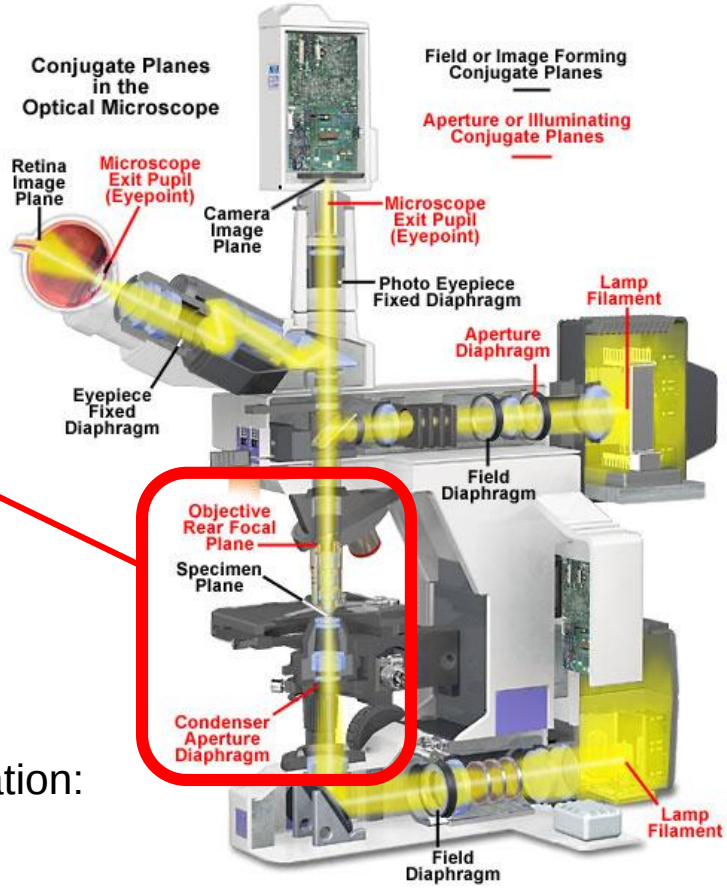
- Magnification
- Image quality – aberrations, alignment, illumination condition etc
- Resolution
- **Contrast**

- **Contrast** is necessary to detect/differentiate details from *background*.
- Captured light from an object must be different in **intensity** or color (= wavelength) from the background light.

Review: Bright-Field (aka Wide-Field) Microscopy

- A basic optical microscopy scheme:





- Illustration shows an upright microscope configuration:
  Here, the illumination is from below.

- **Trade-off between contrast & resolution:**

When the diaphragm is wide open:

NA gets bigger ➔ it leads to HIGHER resolution

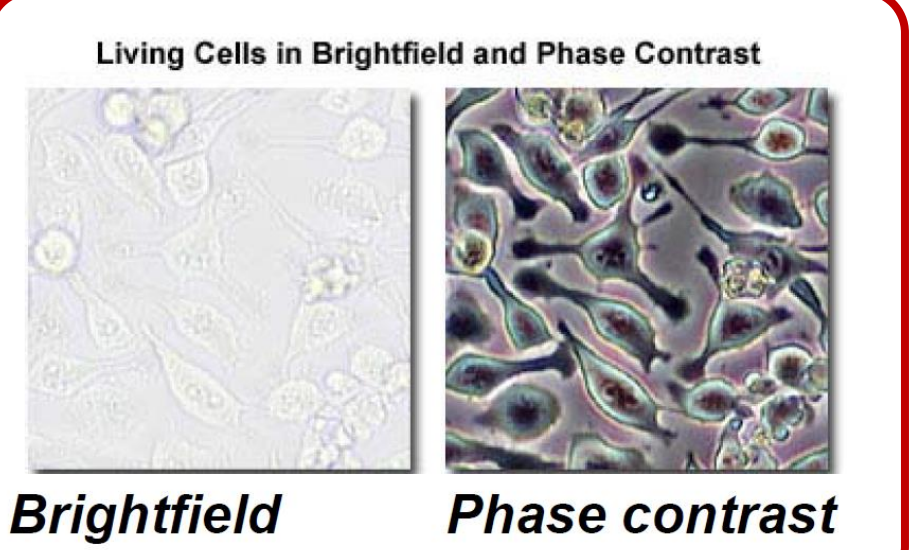
But, as the image gets brighter so does the background ➔ it leads to LOWER contrast

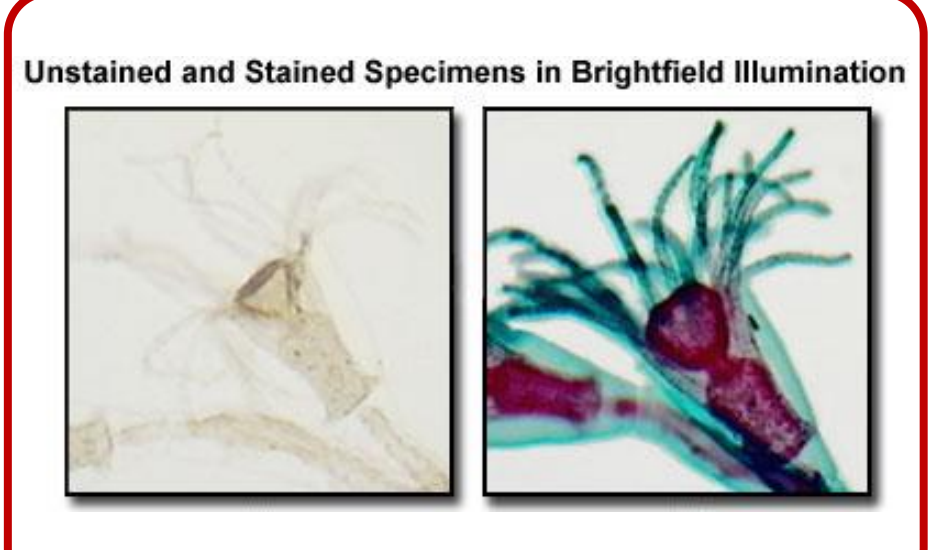
Review: Contrast in Bright-Field Microscope

What is the problem with Bright-Field microcopy?

Most of the biological samples (e.g. cell, tissue etc..) are optically thin and transparent
➔ They do not absorb, scatter → **low sample contrast w.r.t. background**
➔ They are hard to see!!

Contrast depends on background and sample brightness



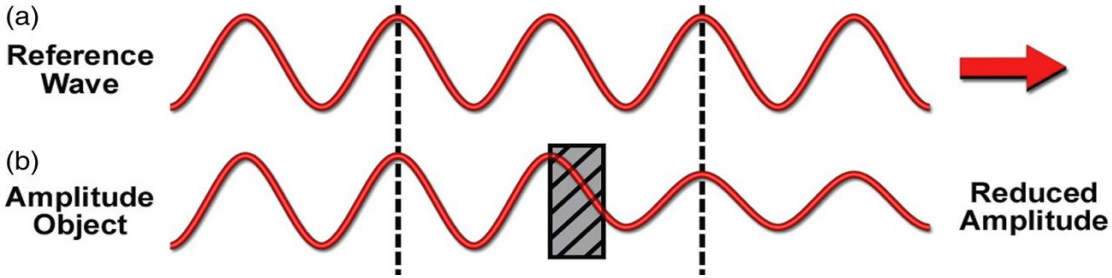


Review: Contrast in microscopy

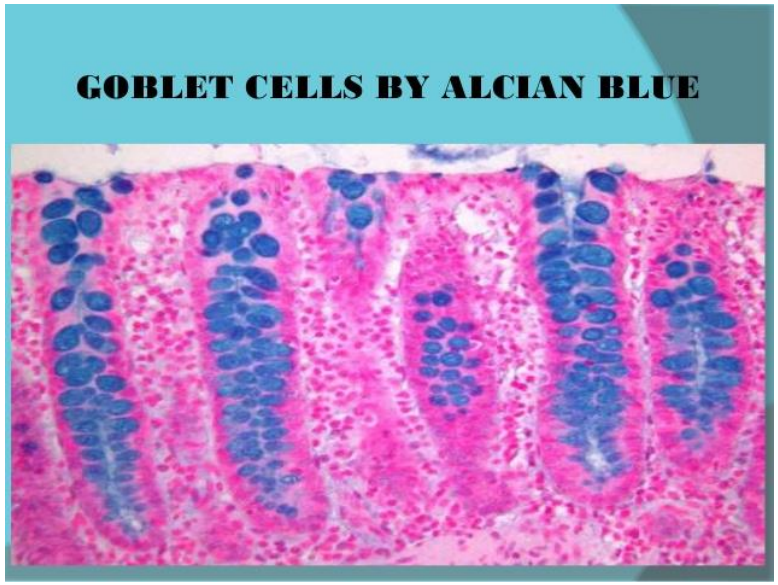
- For transparent specimens, adjust the contrast level:
  - **Bright field microscope** (low contrast)
  - **Stained specimen**
  - Dark field microscopy
  - Phase contrast microscope
  - Polarization microscopy
  - Differential Interference Contrast (DIC) microscopy
  - Fluorescence microscopy

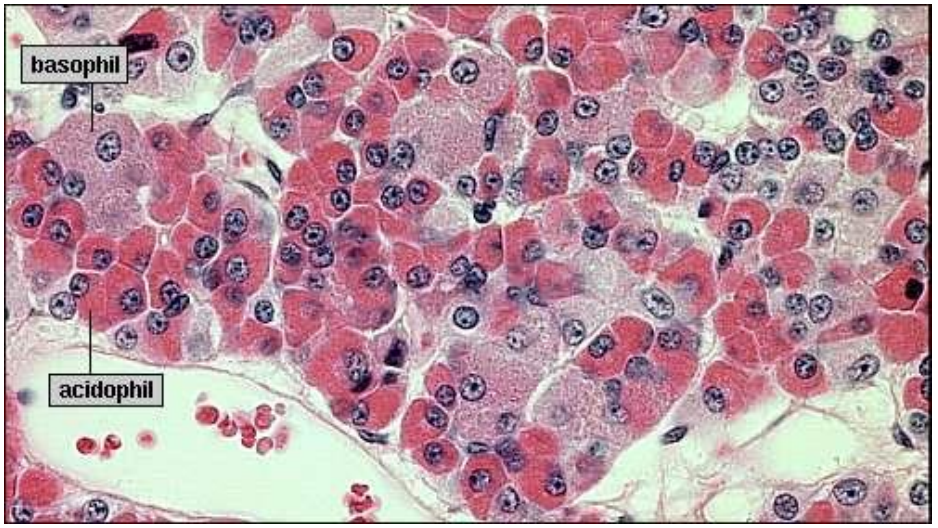
Review: Contrast improves with staining

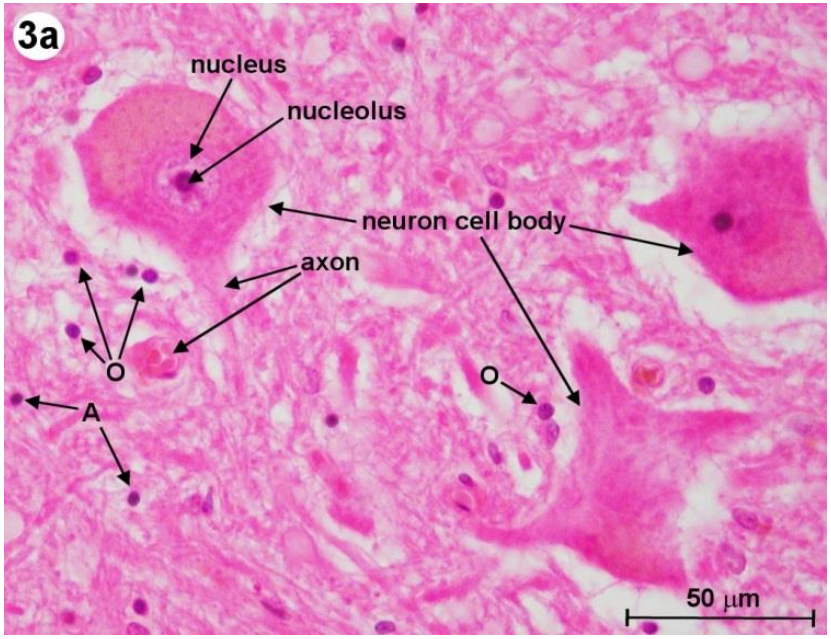
In the case of stained samples, specific wavelengths are absorbed by dyes or pigments.
→This absorption significantly reduces light amplitude, resulting in a high-contrast imaging. Such objects are called **"amplitude objects".**
→ They produce amplitude differences in the image that are directly detected by the eye as differences in intensity.(and also allow objects to appear in color when illuminated with white light).



Review: Staining in biomicroscopy - histology







Contrast in microscopy

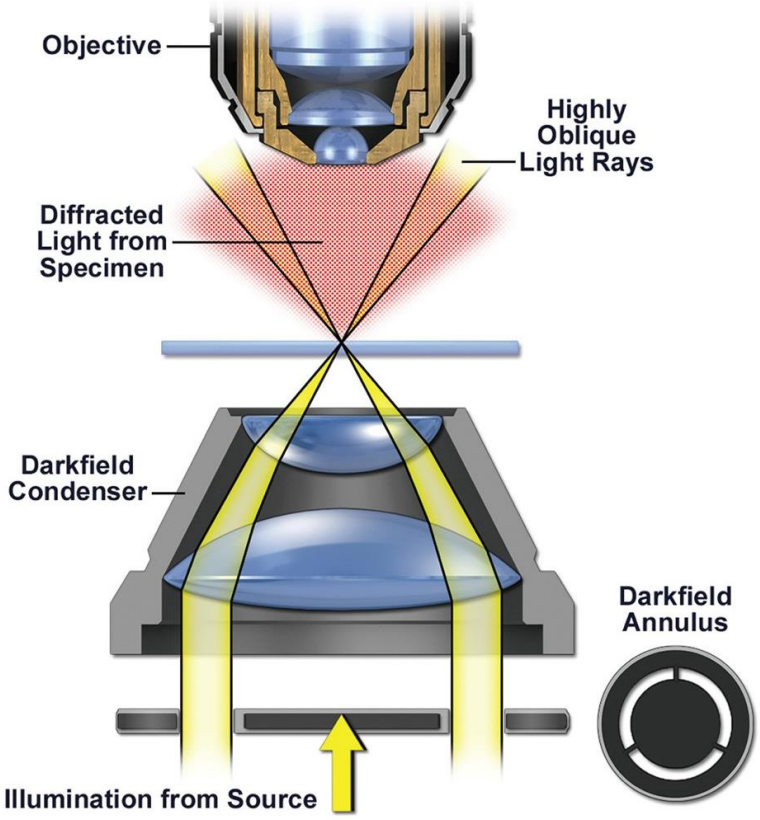
- For transparent specimens, adjust the contrast level:
  - **Bright field microscope** (low contrast)
    - **Stained specimen**
    - **Dark field microscopy**
    - Phase contrast microscope
    - Polarization microscopy
    - Differential Interference Contrast (DIC) microscopy
    - Fluorescence microscopy

Dark Field Microscopy

- Illumination (shown in yellow, bottom left image) is at a steep angle.
- In this way, non-diffracted rays i.e. dominant background signals miss the objective.

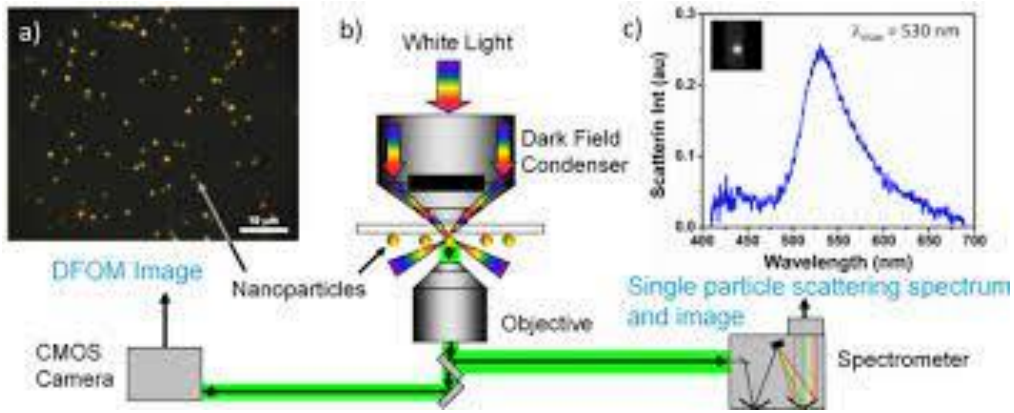
→ This helps to remove background – results in "dark" background

- Only diffracted wave components from the object (shown in red) can make to the objective.



It is a high-contrast technique because:

- Images are based on very small amount of diffracted light from objects.
- Objects are seen clearly against a black (or very dark) background

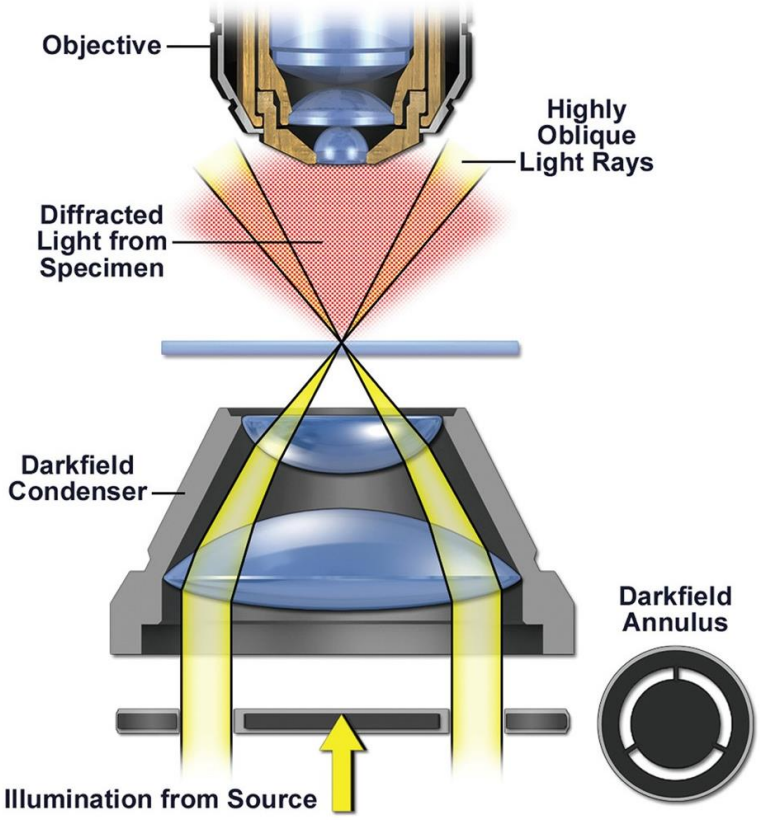


Dark Field Microscopy

- Illumination (shown in yellow, bottom left image) is at a steep angle.
- In this way, non-diffracted rays i.e. dominant background signals miss the objective.

→ This helps to remove background – results in "dark" background

- Only diffracted wave components from the object (shown in red) can make to the objective.



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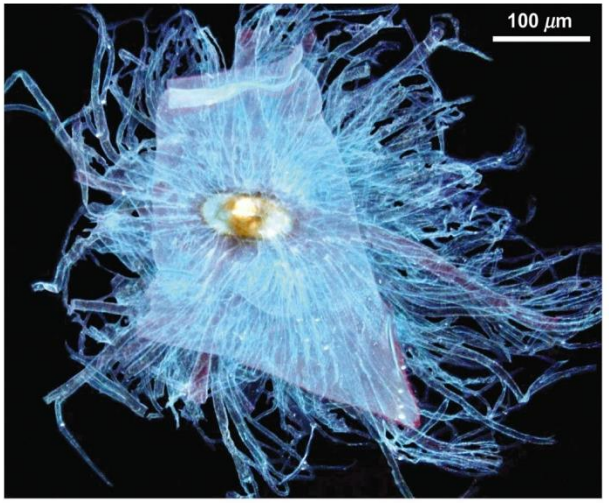
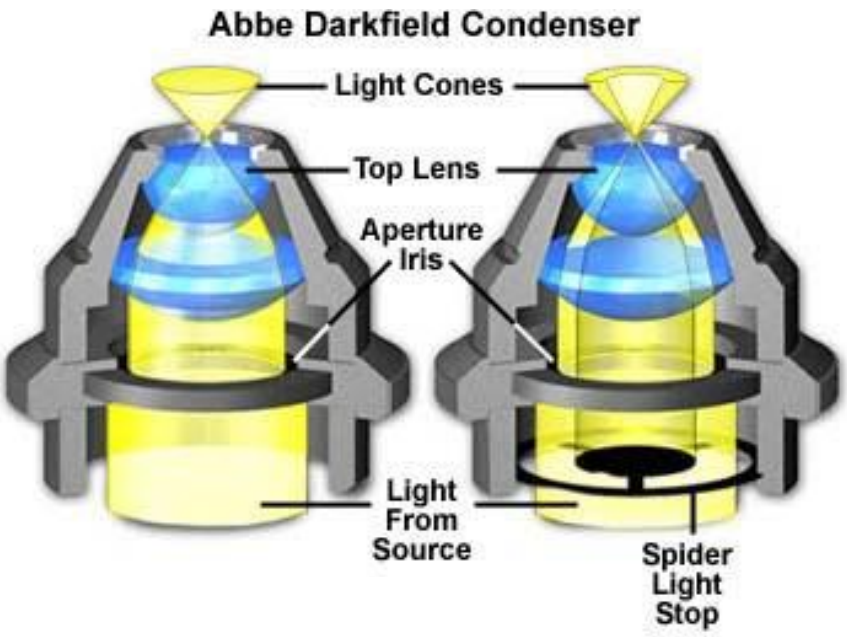


Dark field image of the spiracle (breathing pore) and tracheae of a silkworm

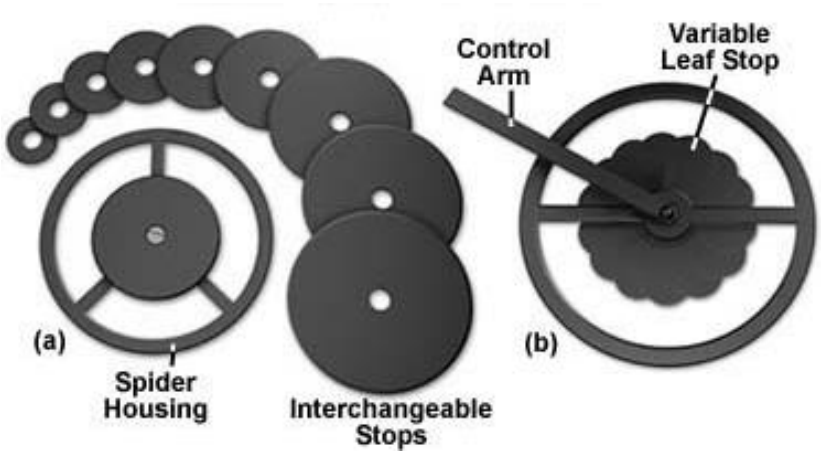
Imaging with Dark Field Microscopy

There are several ways to create dark-field image

1) Use a **dark-field condenser stop** in combination with a medium power lens with NA<0.8.



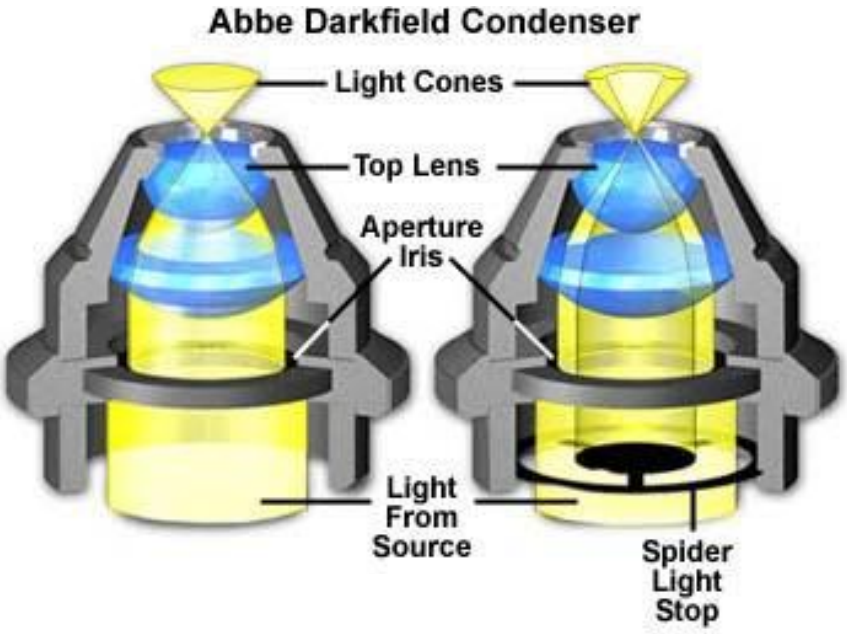
Control of illumination angle & intensity by stops:

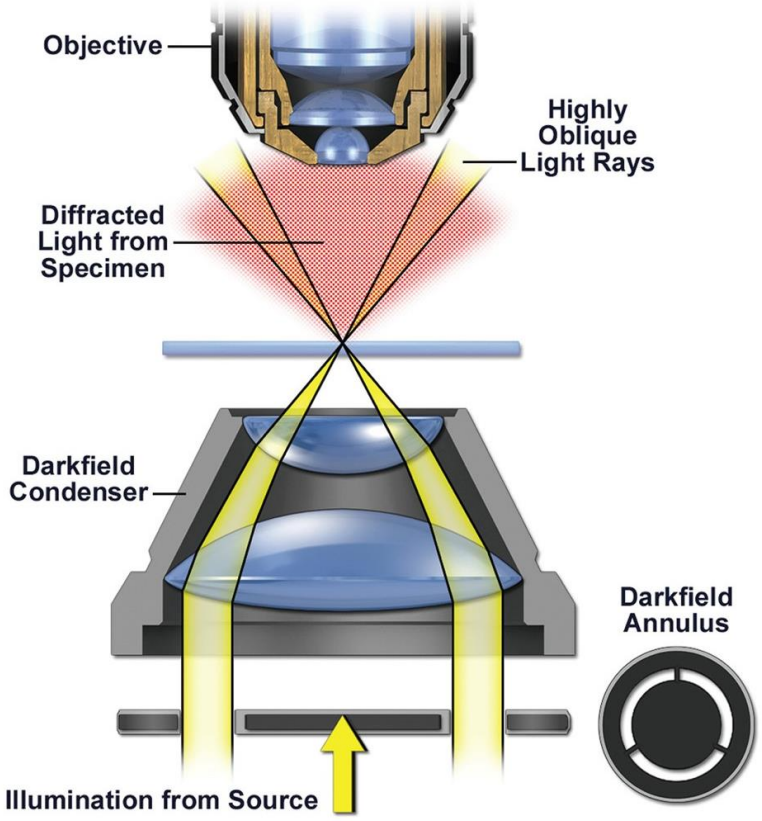


There are several ways to create dark-field image

Option -1:

- Use a **dark-field condenser stop** in combination with a medium power lens with NA<0.8.
- <u>If the NA of the objective is lower than the NA of the illuminating beam generated by the regular condenser plus dark-field spot (annulus), then non-diffracted waves are excluded from the objective.</u>

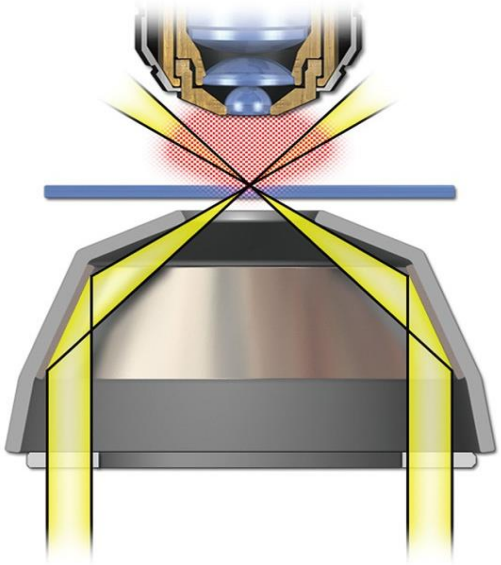




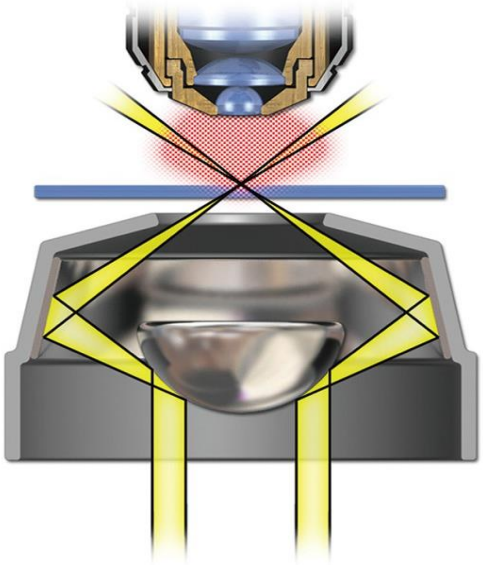
There are several ways to create dark-field image

Option -2:

- For high magnification work requiring oil immersion objectives, one can employ special condensers with parabolic (a) or cardioid (b) reflective surfaces.
- These condensers reflect beams onto the specimen at a very steep angle giving a condenser NA as high as 1.2-1.4.
- The oil immersion objectives used with these condensers can have high NAs (0.9-1.0).

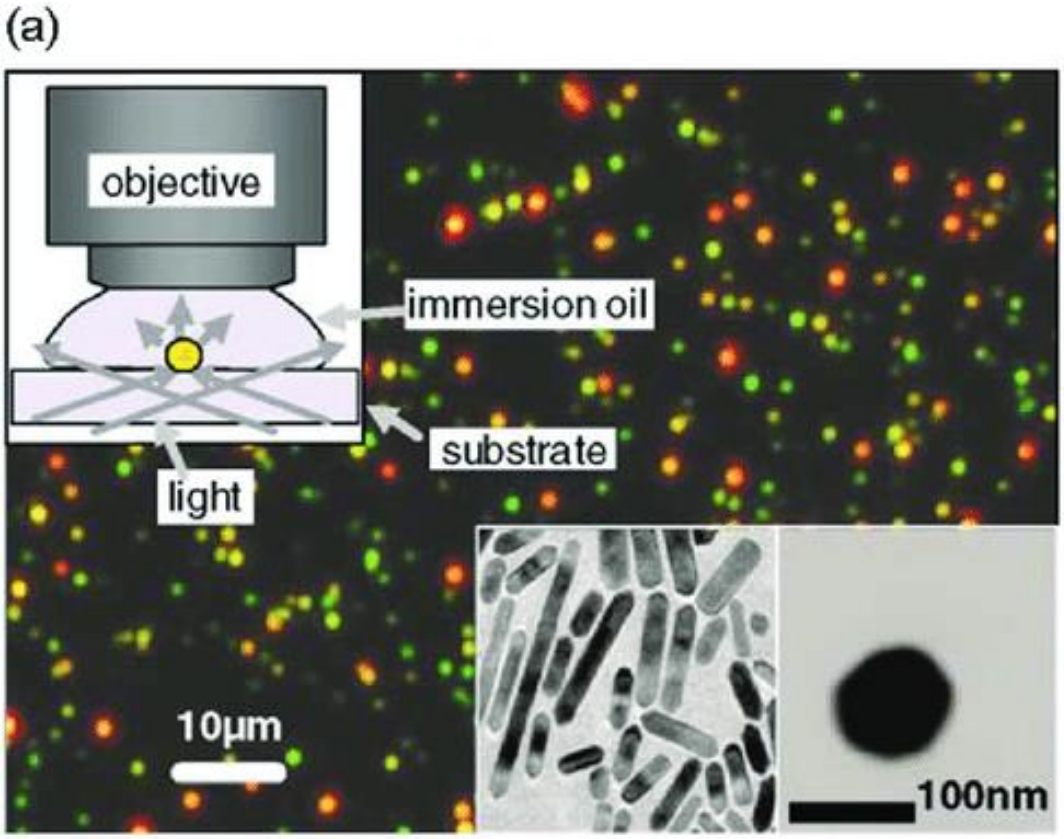


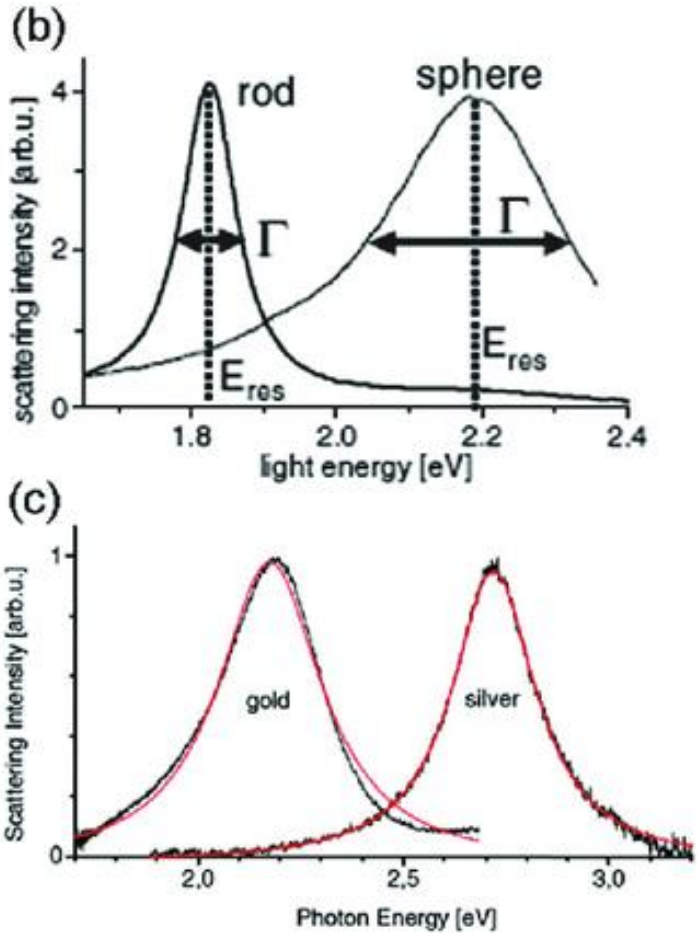
Paraboloid Condenser
(a)



Cardioid Condenser
(b)

Dark Field Microscopy





10.1039/c3cs60367a

Dark Field Microscopy

- Shows sub-resolution details, particles, defects etc. with excellent, reversed contrast
- Good technique for live specimens

- Not for quantitative measurements (i.e. measuring sizes)
- “Detection” term could be more appropriate than “resolution”

➔ Maximizes **detectability**

Rheinberg Illumination

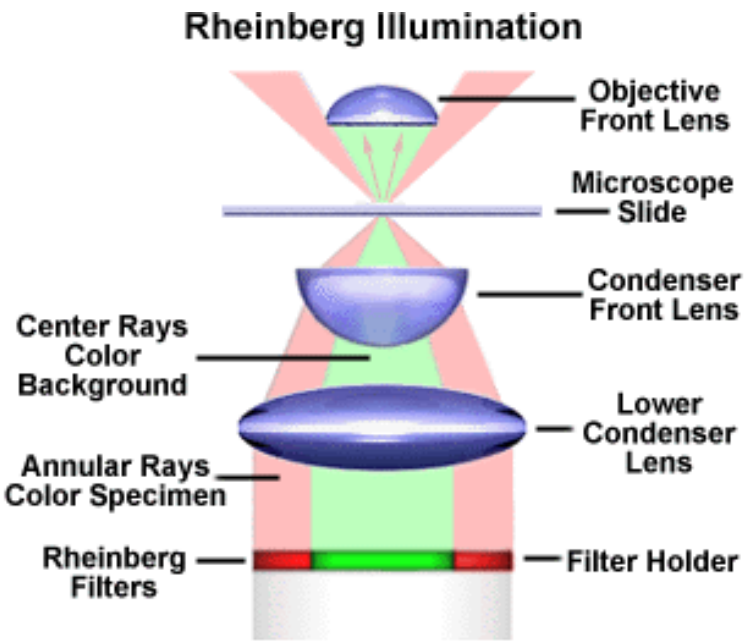
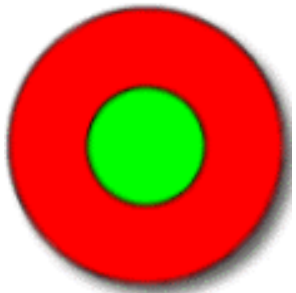


Figure 1

- It is a special variant of dark field illumination
- It uses a **colored dark-field stop** to illuminate the sample with two different colors of light.
  - The central area, where the DF stop would be, is one color (e.g., green)
  - The outer ring (annulus) is a different (contrasting) color (e.g., red).

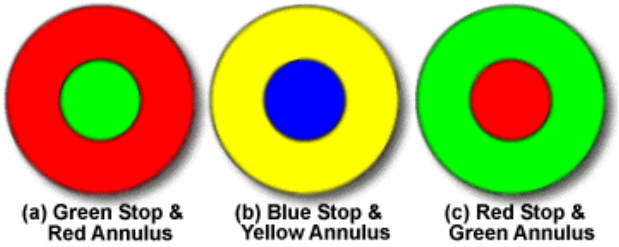
➔**Results in striking color contrast.**

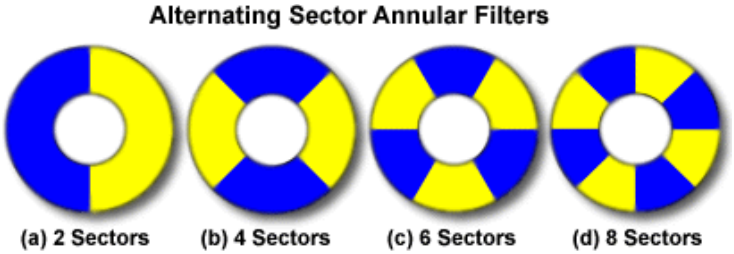

(a) Green Stop & Red Annulus

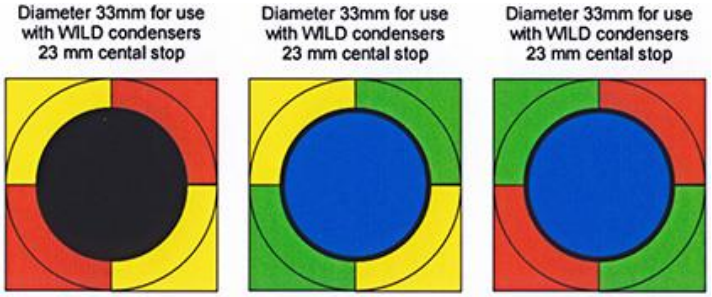
- **Unmodified light** (i.e. light that does not impinge on the sample or non-diffracting) forming the **background** has the color of the **central circle**.
- **Modified** light (i.e. light that impinges on the sample and diffracted) forming the **image** has the color of the **outer annulus**.
- In this example, the sample would be red on a green background.
- Rheinberg stops can be almost any color combination.

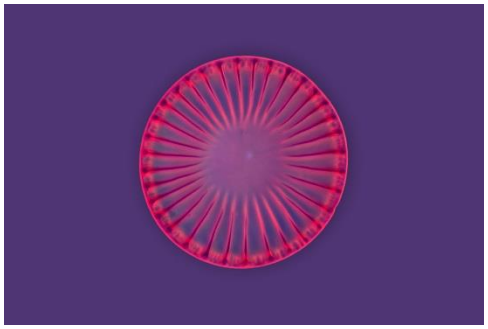
Rheinberg Illumination – art with microscopy

Large variety of filter options:





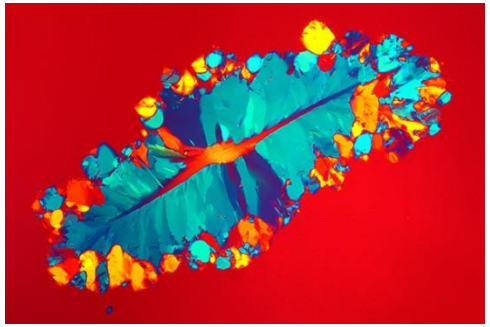




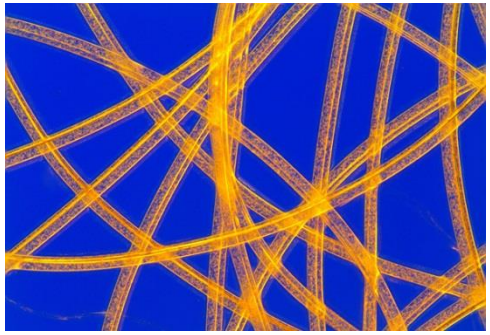
Diatom (algea family)

Bed Bug

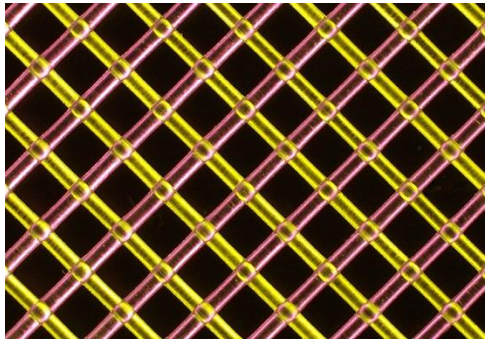
larva of Pleuronectidae



Wine crystal

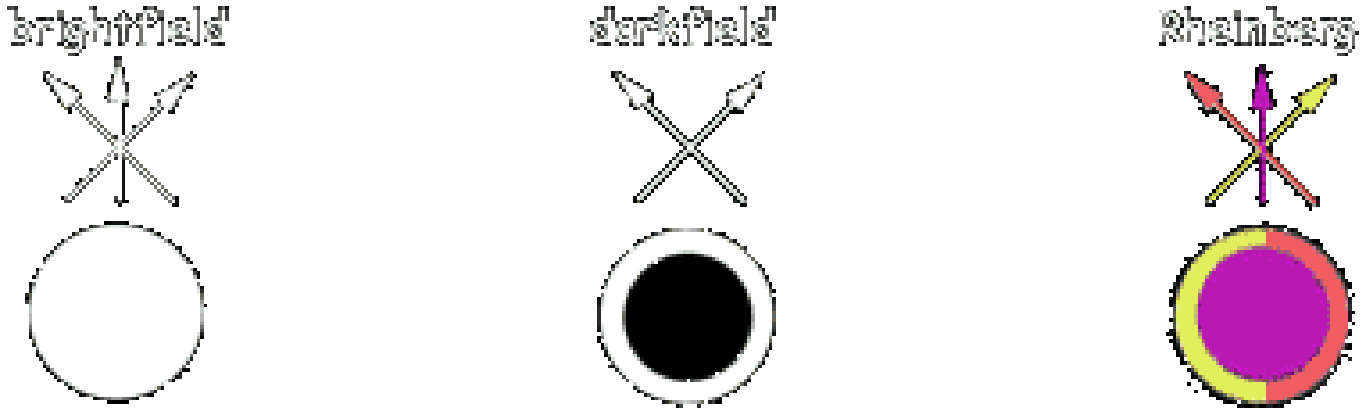


Cat fur



Polyester mesh

Imaging with Dark Field Microscopy



Contrast in microscopy

- For transparent specimens, adjust the contrast level:
  - **Bright field microscope** (low contrast)
  - **Stained specimen**
  - **Dark field microscopy**
  - **Phase contrast microscope**
  - Polarization microscopy
  - Differential Interference Contrast (DIC) microscopy
  - Fluorescence microscopy

Phase in Microscopy

Although most transparent biological samples don't absorb light, they can diffract light & cause phase shift in the rays passing through them ➔ These objects are called "**phase objects**".

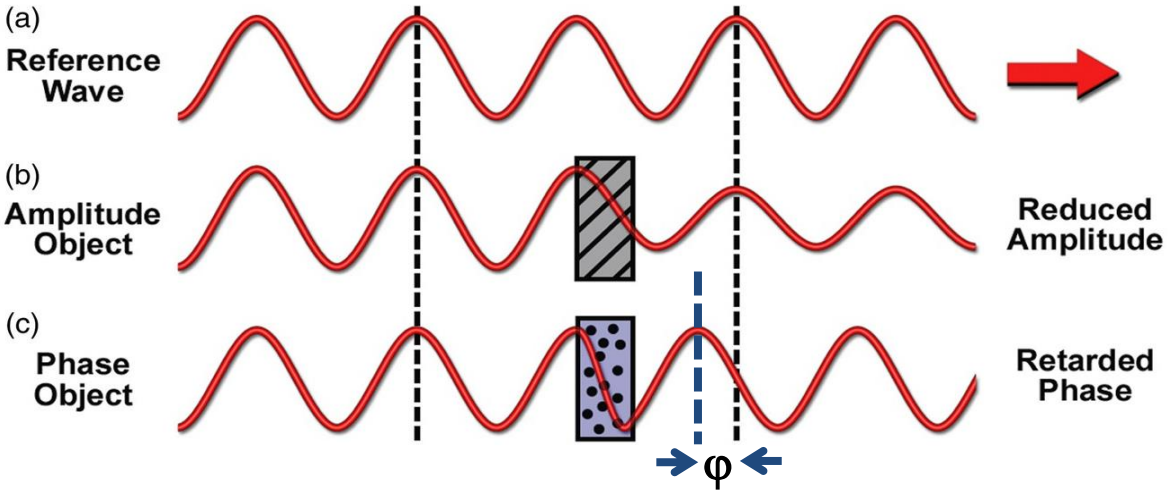
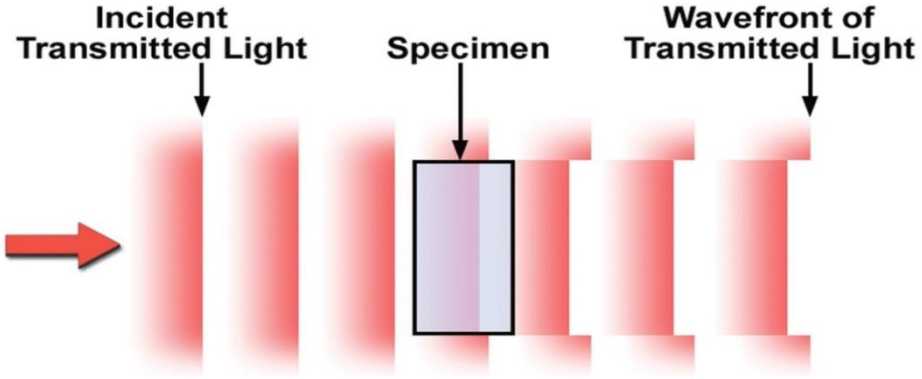


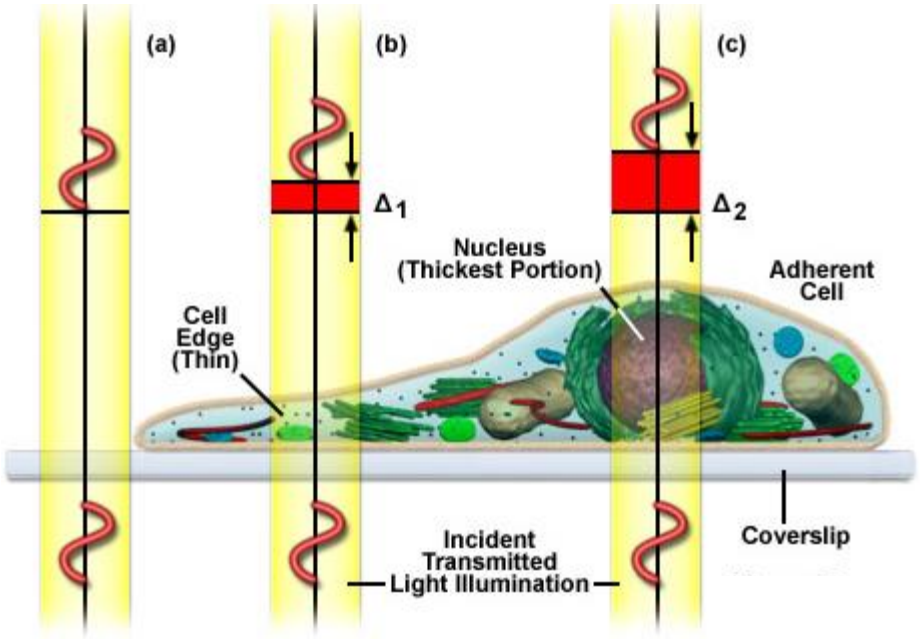
Illustration:
Disturbance of the incident plane wavefront by a phase object



Phase in Microscopy

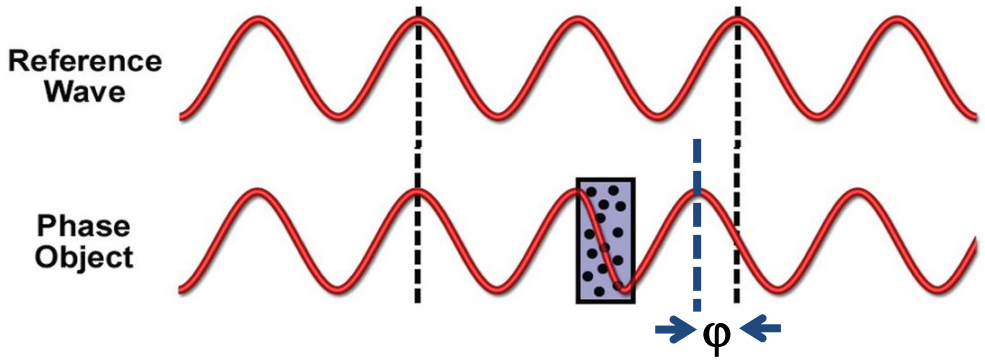
- Cells have higher refractive index (n) than water.
- In higher (n) medium (i.e. cells):
  - Light moves slower (v=c/n)
  - Light has shorter wavelength ($\lambda = \lambda_o/n$)
  - Light is delayed wrt free space

    ➔ **phase-retarded (φ)**



How to exploit this for optical imaging?

Phase Specimens



Surrounding Wave, $\overrightarrow{E_S}$

Particle Wave, $\overrightarrow{E_P}$

retarded in phase

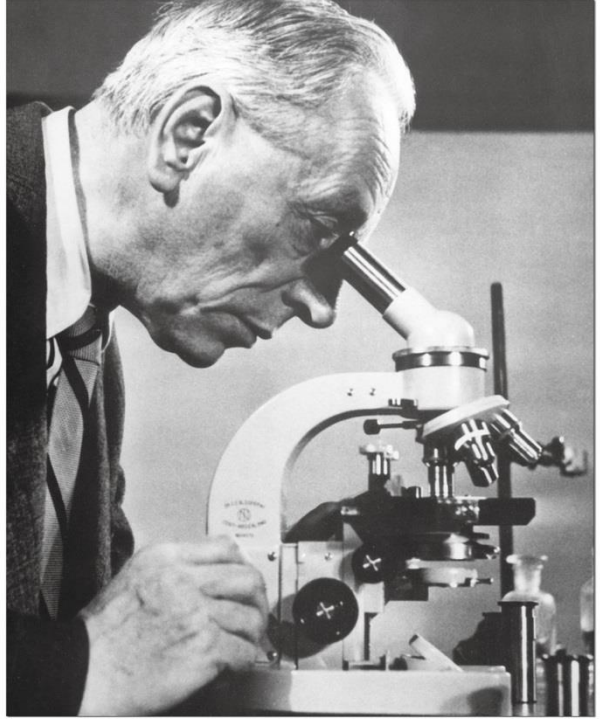
- In biology, most objects are weakly absorbing (i.e. mostly transparent)
- Objects induce a phase shift but detectors (such as eye, cameras) are NOT sensitive to phase, instead detectors are sensitive to amplitude:

$$\overrightarrow{E_S} = 1 \qquad I_S = \left|\overrightarrow{E_S}\right|^2 = 1 \times 1 = 1$$

$$\overrightarrow{E_P} = 1.e^{-i\varphi} \qquad I_P = \left|\overrightarrow{E_P}\right|^2 = 1.e^{-i\varphi} \times 1.e^{+i\varphi} = 1$$

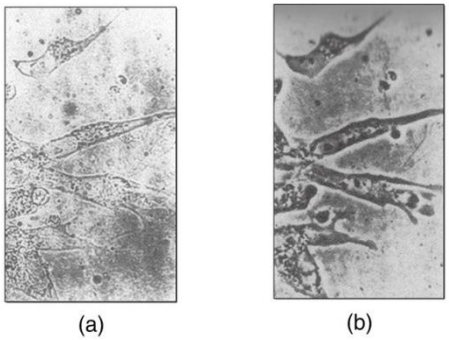
➔ **<u>Consequence</u>: the phase objects are invisible in a standard wide-field (a.k.a bright field) microscopy**

Phase-Contrast Microscopy



In the 1930s, Frits Zernike, a Dutch physicist created an optical design that could transform phase differences to amplitude differences, thus making objects appear as if they had been "optically" stained without any dye and label.

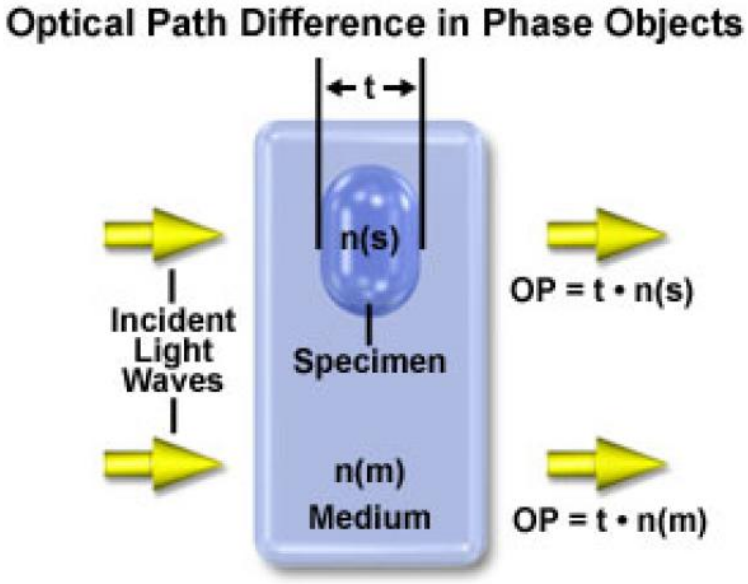
➔ **"Label-free" "high-contrast" microscopy**



Living tissue culture cells as seen with
(a) bright-field
(b) phase contrast

- The Zeiss in Jena introduced **phase contrast objectives & accessories** in 1942, which transformed research in biology and medicine.
- The development of phase contrast microscopy is a brilliant example of how basic research in optics leads to a practical solution for viewing unstained transparent samples.
- For his invention and theory of image formation, Zernike won the Nobel Prize in Physics in 1953.

Optical Path Length and Phase Shift



Optical Path :

$$OP = t \times n$$

Phase shift due to *Optical Path Difference*:

$$\delta = \frac{2\pi}{\lambda}\Delta(OP) = \frac{2\pi}{\lambda}\Delta(OP_s - OP_m)$$

$$= \frac{2\pi}{\lambda}t(n_s - n_m)$$

- ***t*** is the sample thickness → Typical cell in monolayer is ~5μm
- n_s is the refractive index of sample → Most cell has index ~1.36
- n_m is the refractive index of medium → Cell medium index is ~1.335

Calculate optical path difference: Ans: ~125 nm

For visible light illumination with λ_{green} ~ 500 nm

- What is the ratio of optical path difference to wavelength ? Ans: $\lambda/4$
- What is the amount of phase shift ? Ans: $\pi/2$ or 90^o