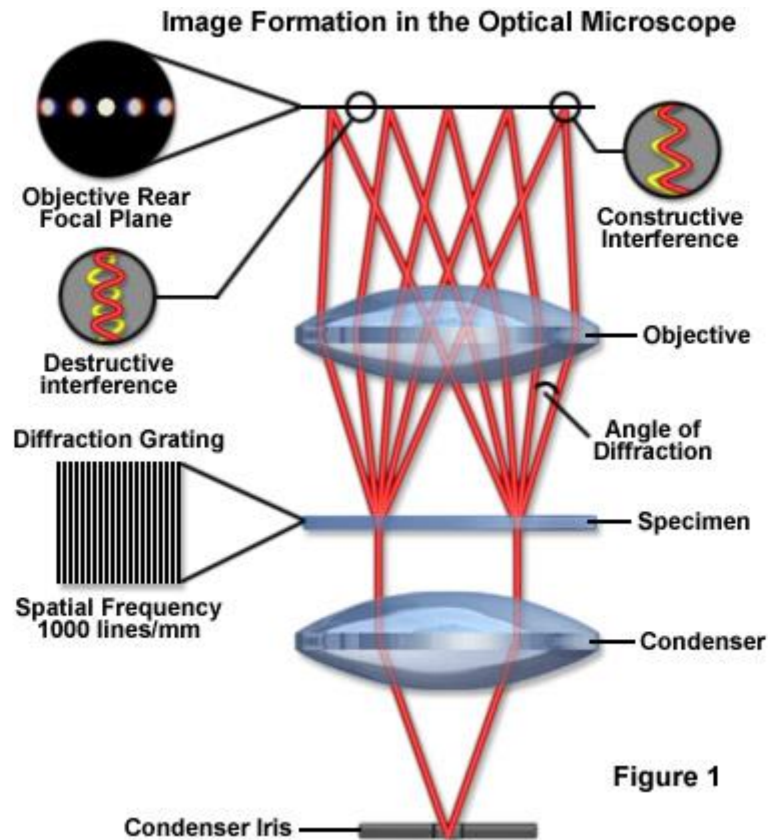
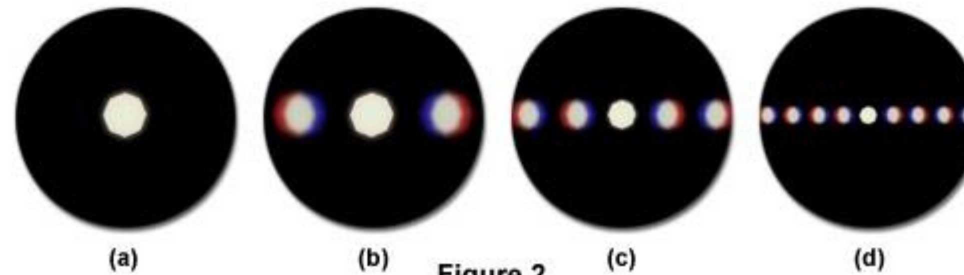
# Transmitted light microscopy

PG Sem 1 (BIOS0701)

# Light microscopy




Direct, undeviated, or non-diffracted light
  background light
Deviated and diffracted light
  passes through opaque part
    destructive interference



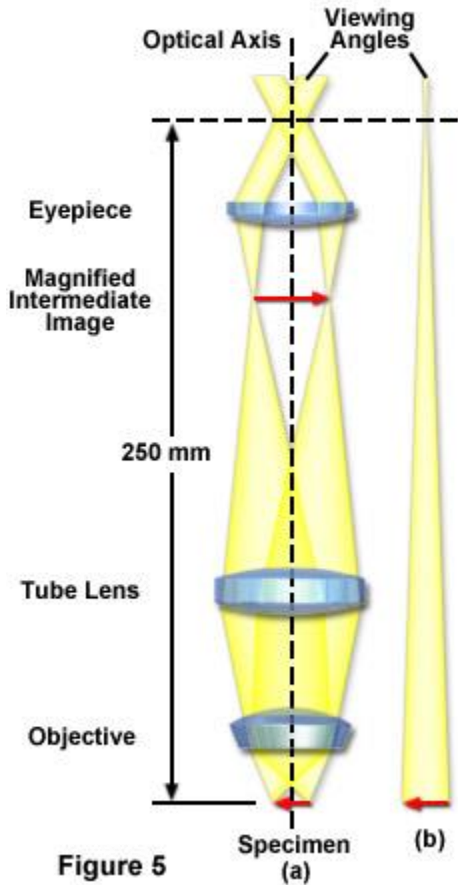
# Basic compound microscope

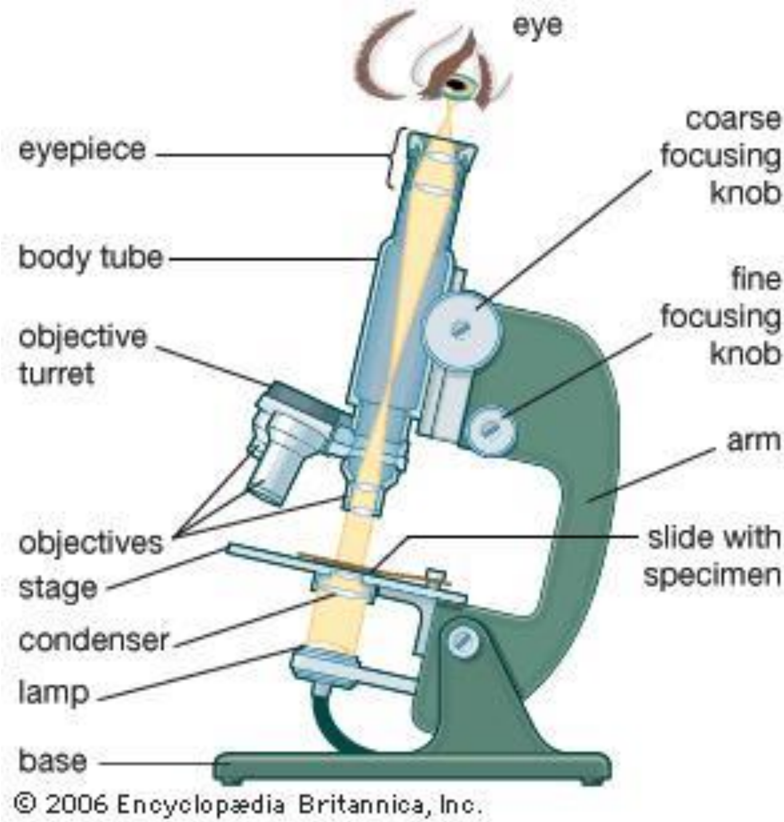
Monocular

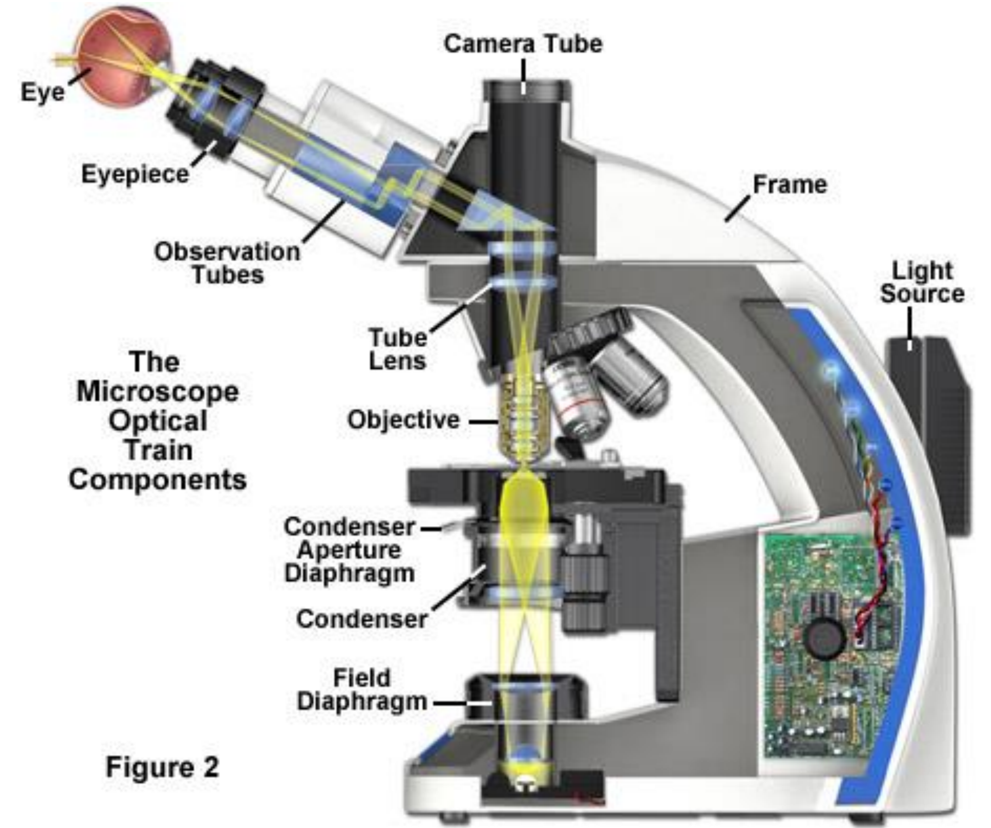
Binocular
Upright

Binocular
Inverted

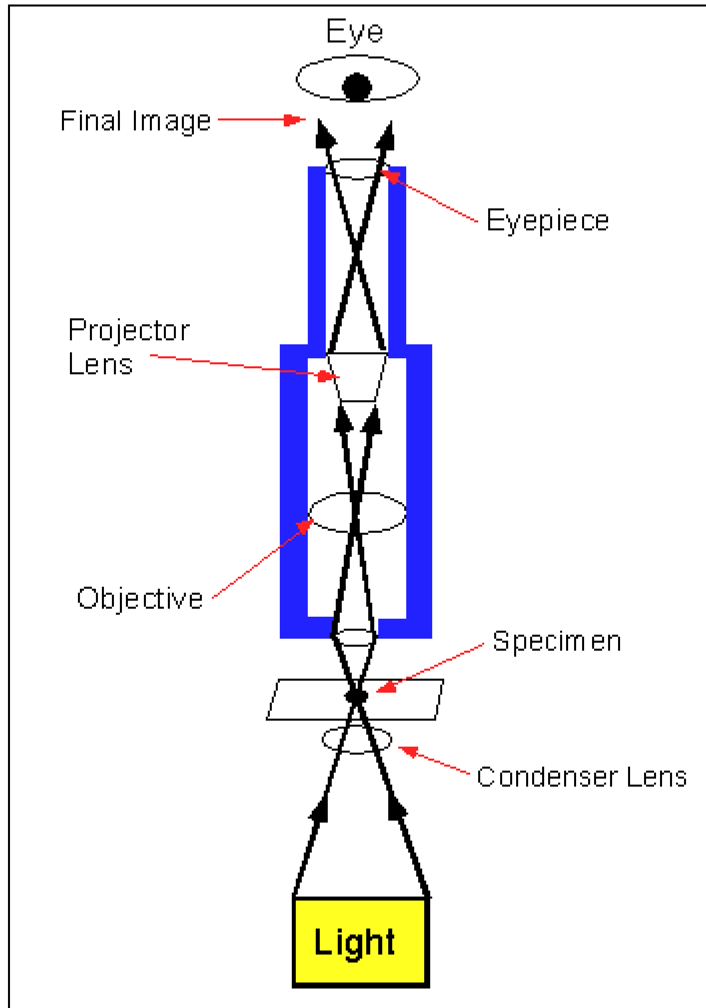
# Light path



# Illumination (Köhler)




Conjugate Focal Planes in the Microscope for Köhler Illumination

Conjugate Field Planes

Conjugate Aperture Planes

Retina

Eye

Iris Diaphragm of the Eye

Intermediate Image

Eyepiece

Field Stop

Rear Focal Plane of Objective

Objective

Object (Specimen) Plane

Slide

Condenser Lens

Front Focal Plane of Condenser

Field Stop Diaphragm

Collector Lens

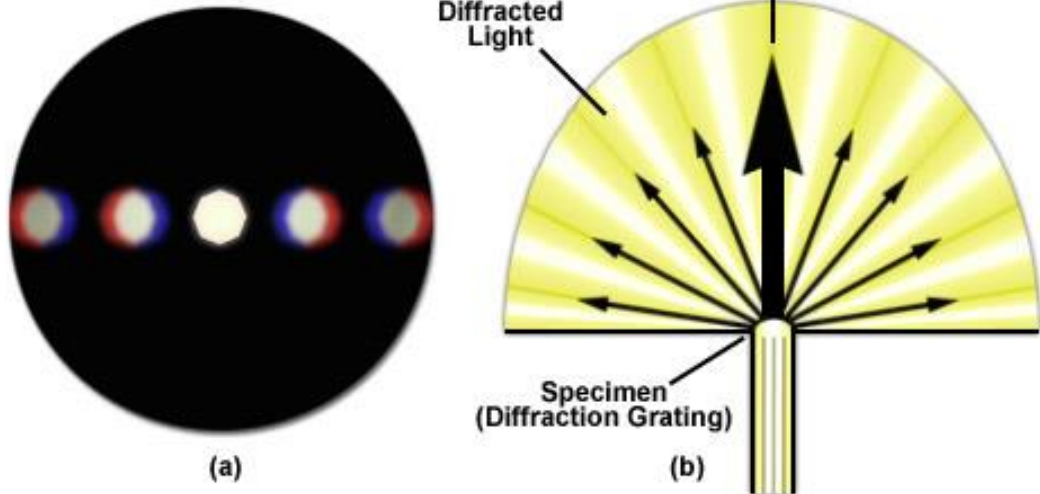
Lamp

Lamp Filament

Figure 1

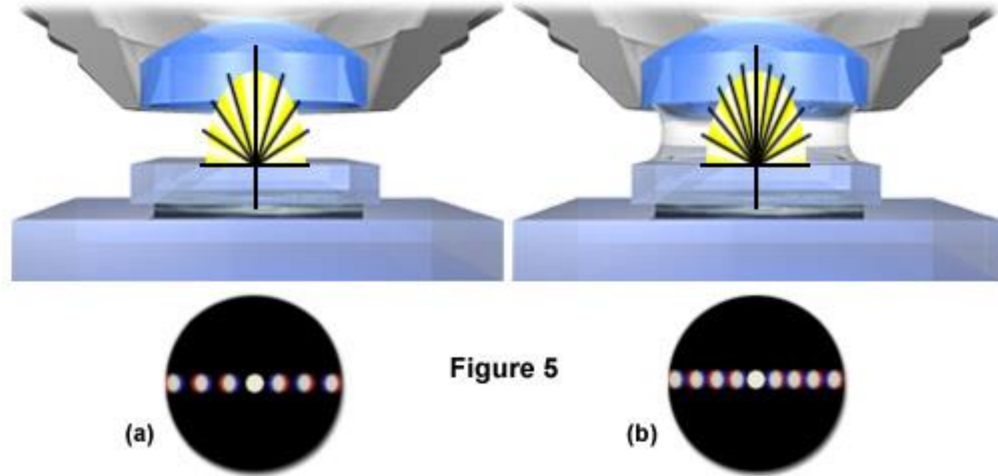
Closing or opening the condenser diaphragm controls the angle of the light rays emerging from the condenser and reaching the specimen from all azimuths. Because the light source is not focused at the level of the specimen, the light at specimen level is essentially grainless and extended, and does not suffer deterioration from dust and imperfections on the glass surfaces of the condenser.

# Diffraction




- The central spot of light (image of the condenser aperture diaphragm) represents the direct or undeviated light passing through the specimen or around the specimen undisturbed
  - $0^{th}$ or zeroth order
- The fainter images are $1^{st}$, $2^{nd}$ $3^{rd}$ etc.
- The blue wavelengths are diffracted at a lesser angle than the green wavelengths, which are diffracted at a lesser angle than the red wavelengths. At the rear focal plane of the objective, the blue wavelengths from each slit interfere constructively to produce the blue area of the diffracted image of each spectrum or order. The red and green areas are spaced a bit further, but arise from the same phenomenon.

# Diffracted Light and Resolution



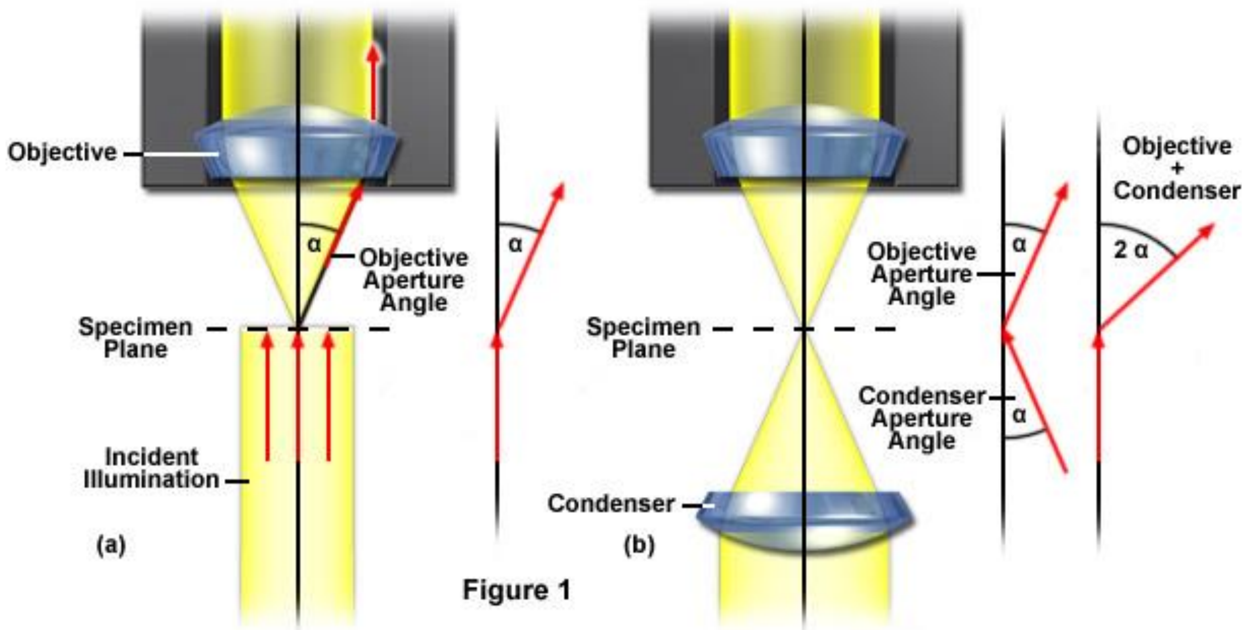

Microscope specimens can be considered as complex line or pattern gratings with details and openings spanning a large range of sizes.

According to Ernst Abbe, the details of a specimen will be resolved if the objective captures 2 orders of light, such as the $0^{th}$ order of the light and at least the $1^{st}$ order of diffraction.

The greater the number of diffracted orders that gain admittance to the objective, the more accurately the image will represent the original object.

- So, a medium with higher refractive index than air (such as immersion oil) will capture more diffracted orders and yield better resolution than a dry objective.

# Numerical aperture and resolution

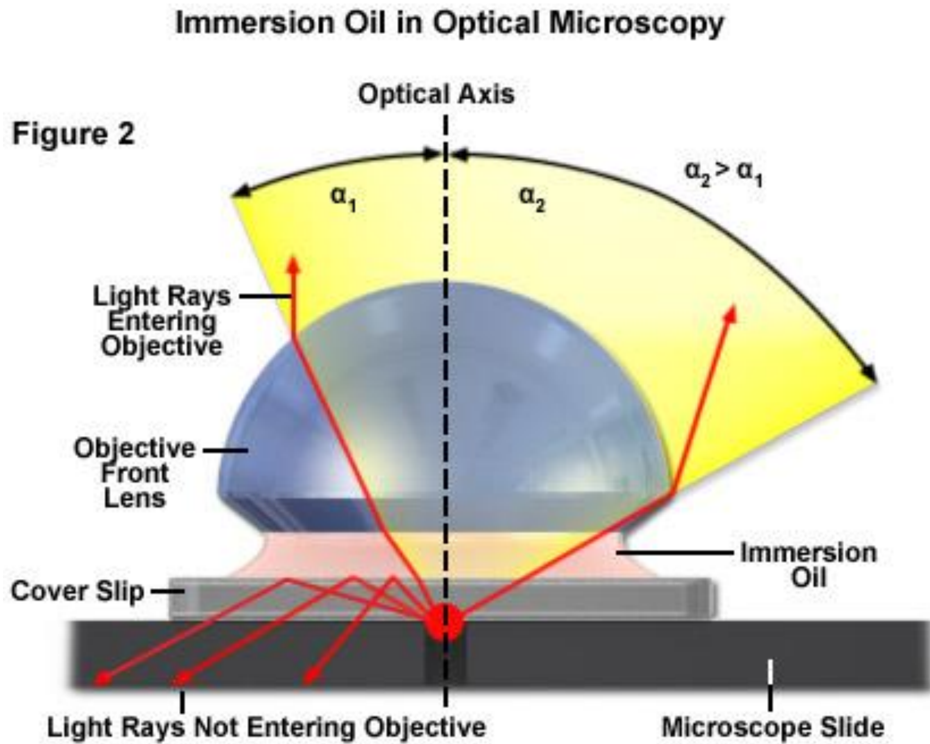



- If small objects (such as a typical stained specimen mounted on a microscope slide) are viewed through the microscope, the light incident on these minute objects is diffracted so that it deviates from the original direction.
- In order to increase the effective aperture and resolving power of the microscope, a condenser (Figure 1(b)) is added to generate a ray cone on the illumination side of the specimen.

- To quantitate the difference **Numerical Aperture (NA) = $\eta \cdot \sin(\alpha)$**
  - where **$\alpha$** equals one-half of the objective's opening angle and **$\eta$** is the refractive index of the immersion medium

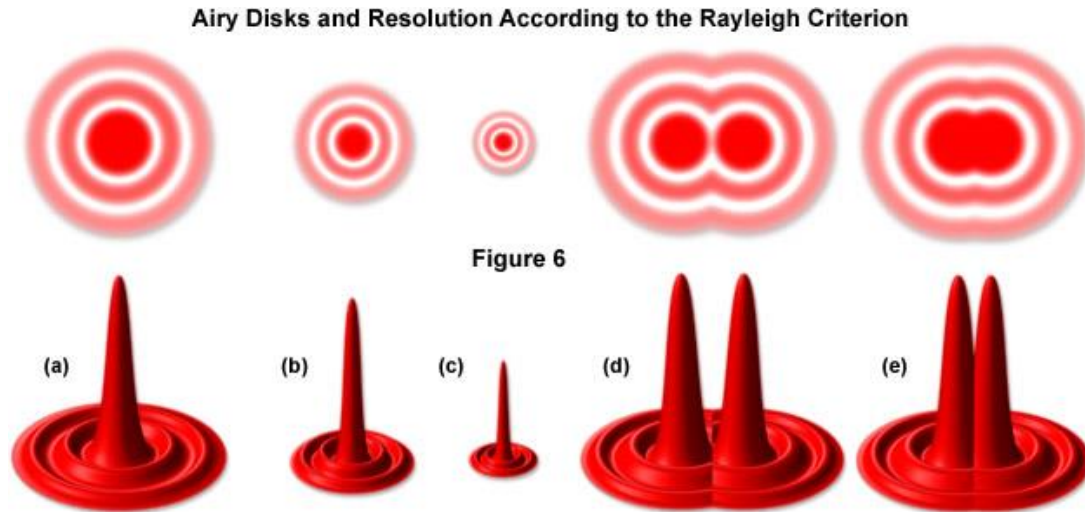
# Immersion media

- Refractive index of glass is 1.51
- So the immersion oils generally have same refractive index



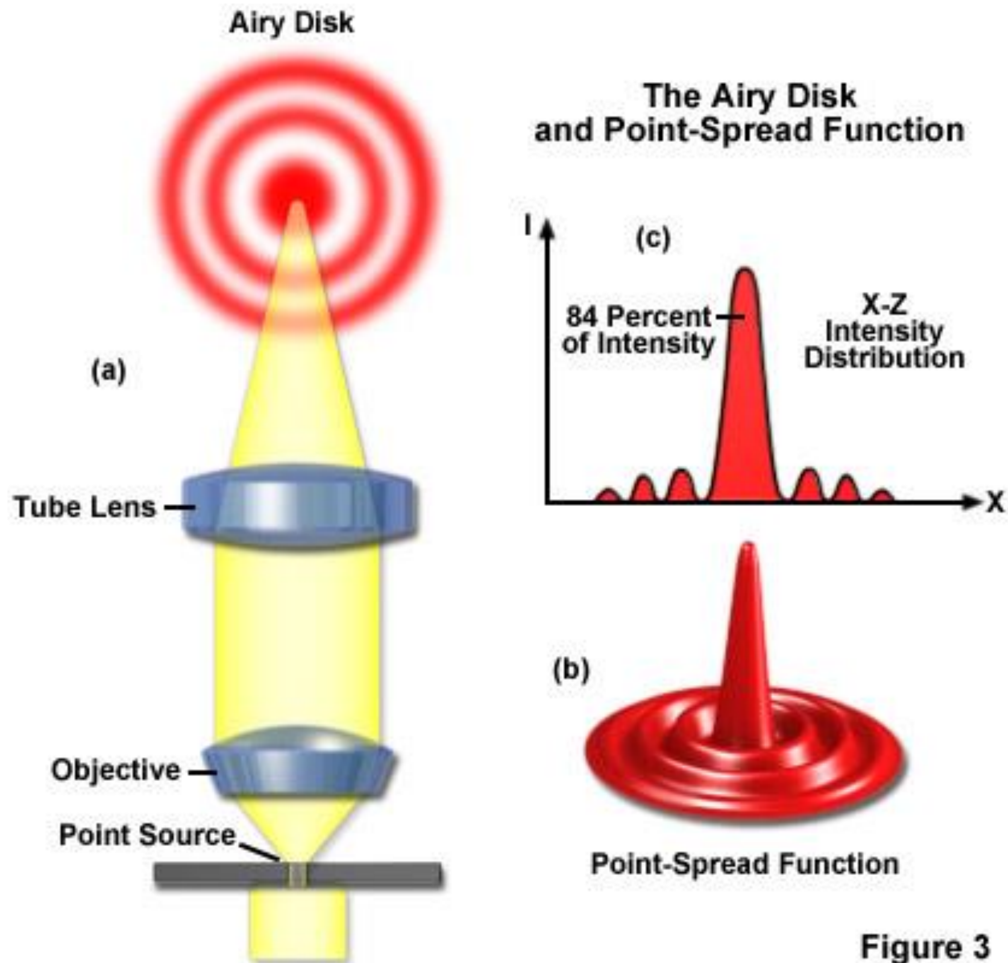

| Magnification | Plan Achromat (NA) | Plan Fluorite (NA) | Plan Apochromat (NA) |
|---|---|---|---|
| 0.5x | 0.025 | n/a | n/a |
| 1x | 0.04 | n/a | n/a |
| 2x | 0.06 | 0.08 | 0.10 |
| 4x | 0.10 | 0.13 | 0.20 |
| 10x | 0.25 | 0.30 | 0.45 |
| 20x | 0.40 | 0.50 | 0.75 |
| 40x | 0.65 | 0.75 | 0.95 |
| 40x (oil) | n/a | 1.30 | 1.40 |
| 63x | 0.75 | 0.85 | 0.95 |
| 63x (oil) | n/a | 1.30 | 1.40 |
| 100x (oil) | 1.25 | 1.30 | 1.40 |

# Airy disks and resolution



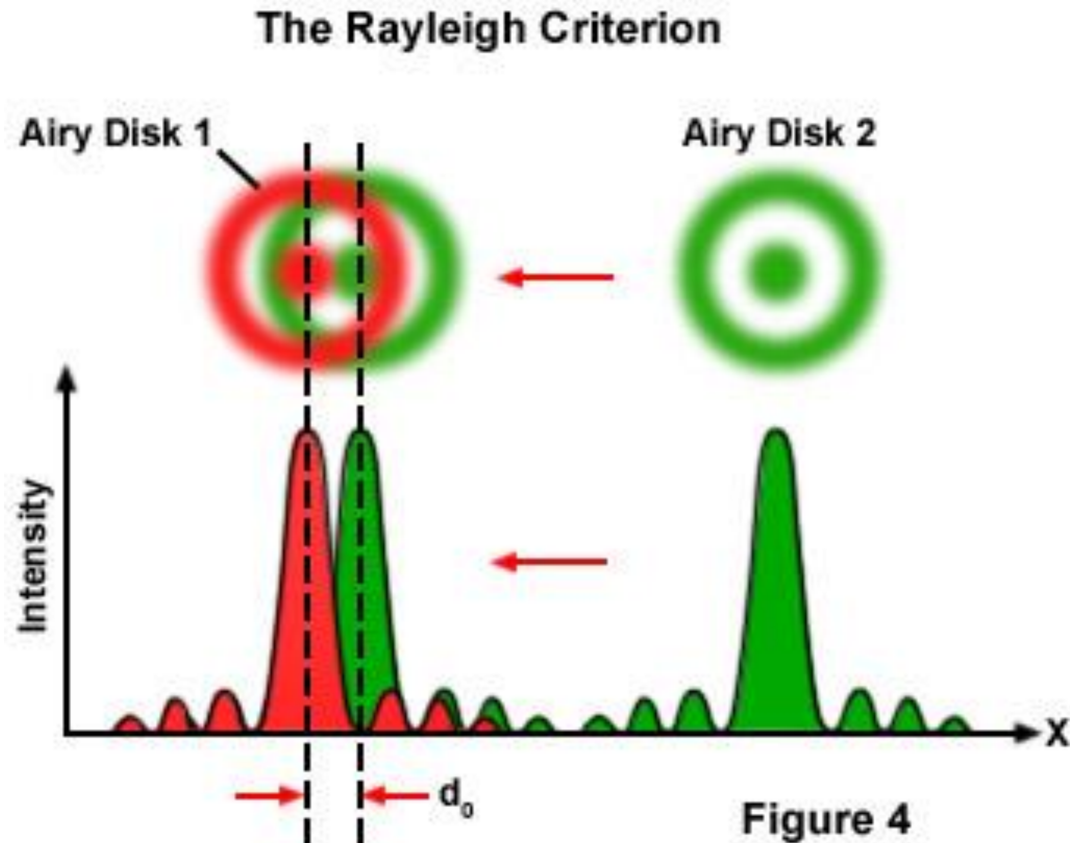

- For small details in a specimen (as opposed to a line grating), the objective projects the direct and diffracted light onto the image plane of the eyepiece diaphragm in the form of small, circular diffraction patterns known as Airy disks. High numerical aperture objectives capture more of the diffracted orders and produce smaller size disks than do low numerical aperture objectives.
- In Figure 6, Airy disk size is shown steadily decreasing from Figure 6(a) through Figure 6(c). The larger disk sizes in Figures 6(a) and (b) are produced by objectives with lower numerical aperture, while the very sharp Airy disk in Figure 6(c) is produced by an objective of very high numerical aperture.

# Airy disk and point spread function




- Three-dimensional representations of the diffraction pattern near the intermediate image plane are known as the point-spread function
- $\text{Resolution}_{x,y} = \lambda / 2[\eta \cdot \sin(\alpha)] = \lambda / 2\ NA$
- $\text{Resolution}_{z} = 2\lambda / [\eta \cdot \sin(\alpha)]^2 = 2\lambda / NA^2$
- the theoretical resolution limit at the shortest practical wavelength (approximately 400 nm) is around 150 nm in the lateral dimension and approaching 400 nm in the axial dimension when using an objective having a numerical aperture of 1.40

# Rayleigh Criterion



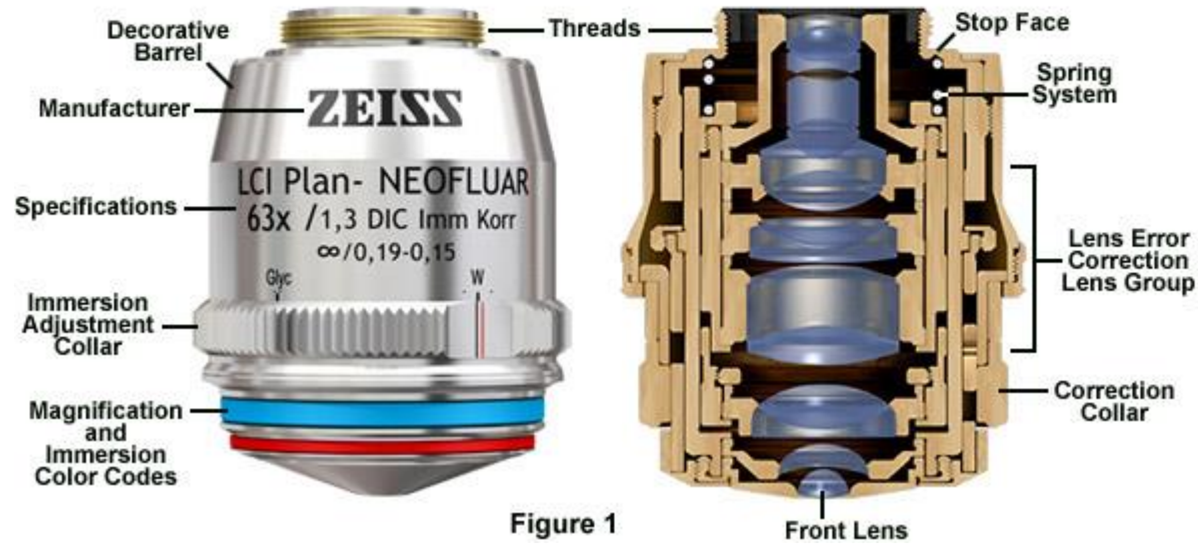

- Consider two Airy disks where the intensity profiles have been superimposed. If the two image points are far away from each other, they are easy to recognize as separate objects. However, when the distance between the Airy disks is increasingly reduced, a limit point is reached when the principal maximum of the second Airy disk coincides with the first minimum of the first Airy disk. The superimposed profiles display two brightness maxima that are separated by a valley. The intensity in the valley is reduced by approximately 20 percent compared with the two maxima. This is just sufficient for the human eye to see two separate points, a limit that is referred to as the **Rayleigh criterion**.

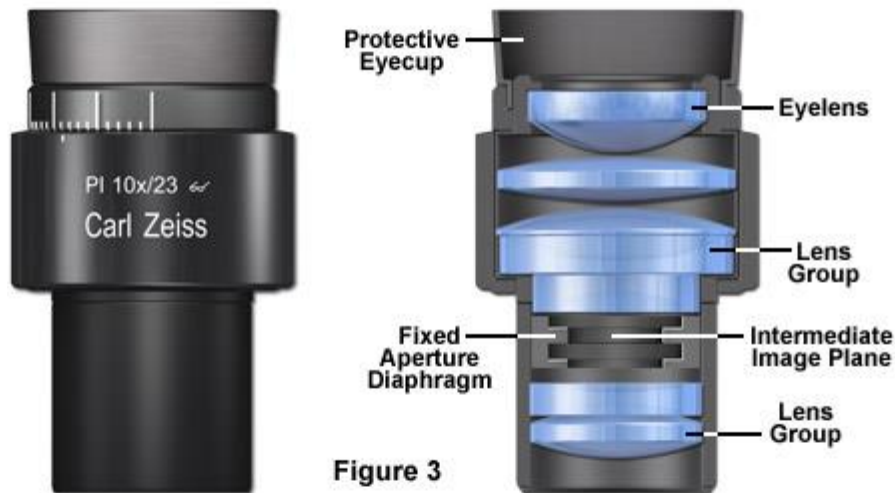
**1.22 is a calculated factor based on the example**

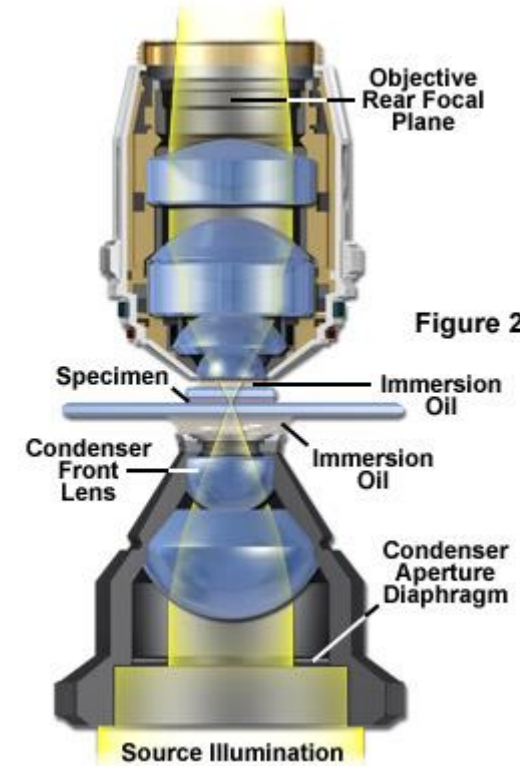
# Resolution

- Rayleigh equation often cited as the basis for calculating point-to-point resolution in the microscope
  - **d (resolution) = 1.22 • (λ/2NA)**
- λ is necessary because blue light is diffracted at a lesser angle than green light or red light, a lens of a given aperture may capture more orders of light when the wavelengths are in the blue region of the visible light spectrum.
- NA = Numerical Aperture. The greater the number of higher diffracted orders admitted into the objective, the smaller the details of the specimen that can be clearly separated or resolved. Herein is the value of using high numerical aperture objectives for examining the smallest possible details in various specimens.
- Also the shorter the wavelength of visible light used, the better the resolution.
- Keep in mind why high numerical aperture, apochromatic lenses can separate extremely small details in blue light.

# Opticals



# Aberrations

- Spherical

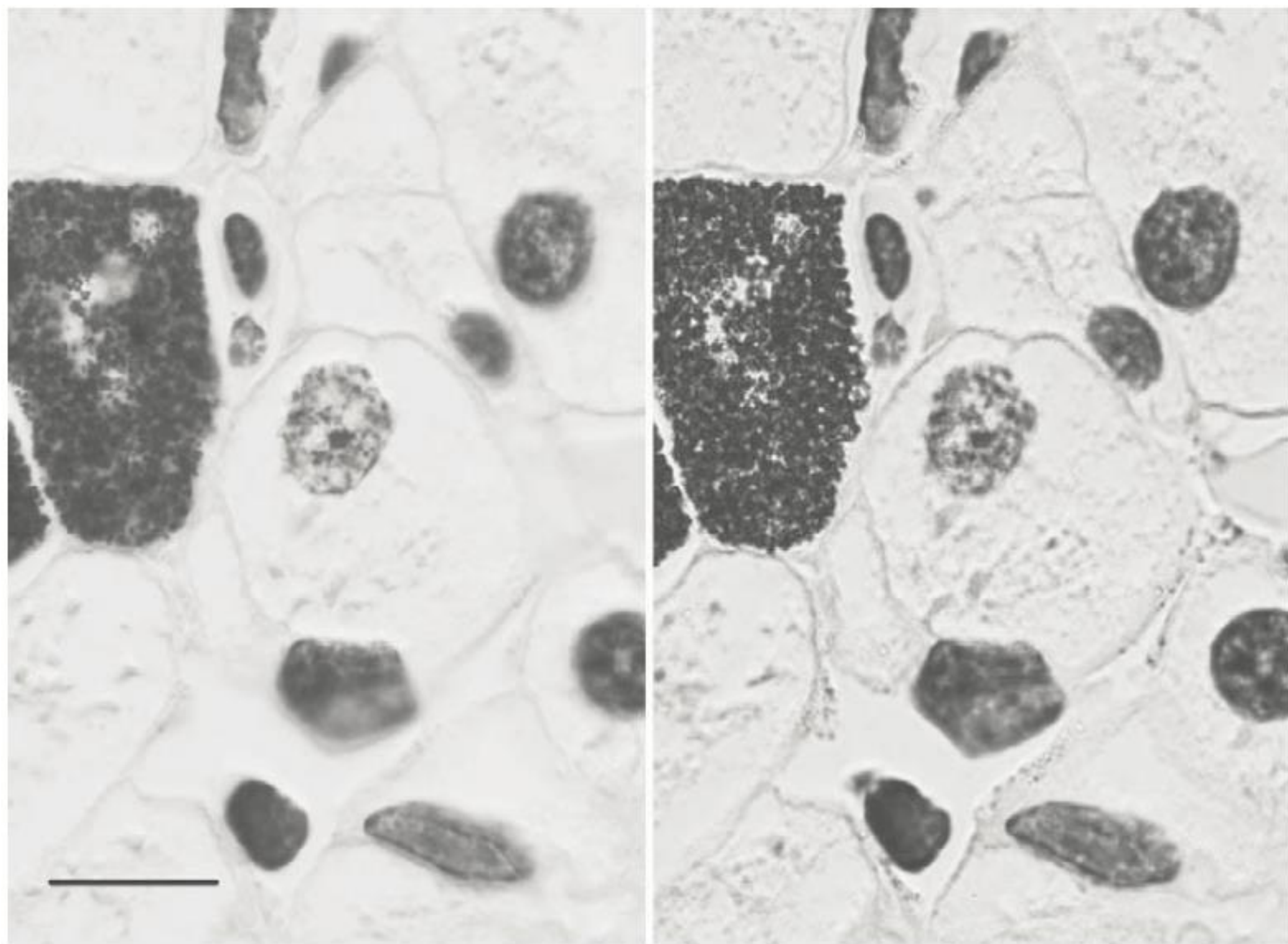
- Chromatic

| Objective Specification | Spherical Aberration | Chromatic Aberration | Field Curvature |
|---|---|---|---|
| Achromat | 1 Color | 2 Colors | No |
| Plan Achromat | 1 Color | 2 Colors | Yes |
| Fluorite | 2-3 Colors | 2-3 Colors | No |
| Plan Fluorite | 3-4 Colors | 2-4 Colors | Yes |
| Plan Apochromat | 3-4 Colors | 4-5 Colors | Yes |

# Contrast

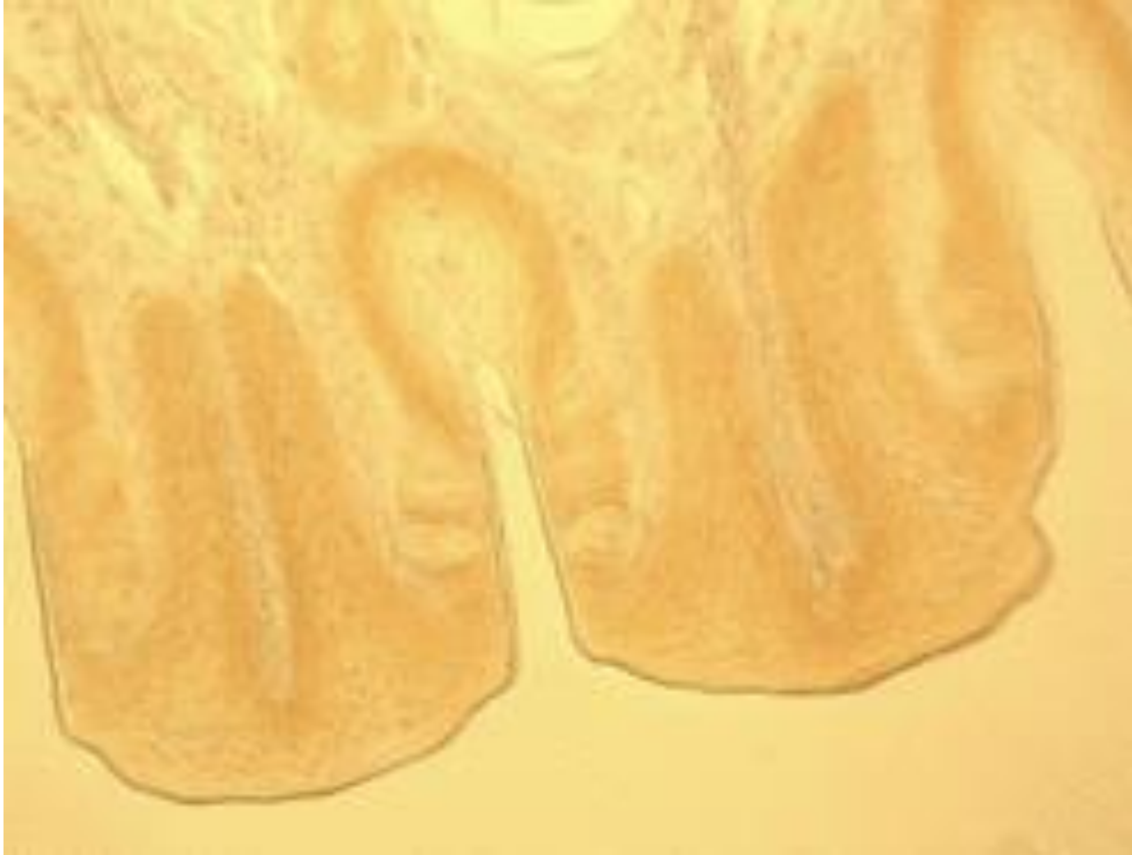
Is the name of the game

# Resolution vs Contrast

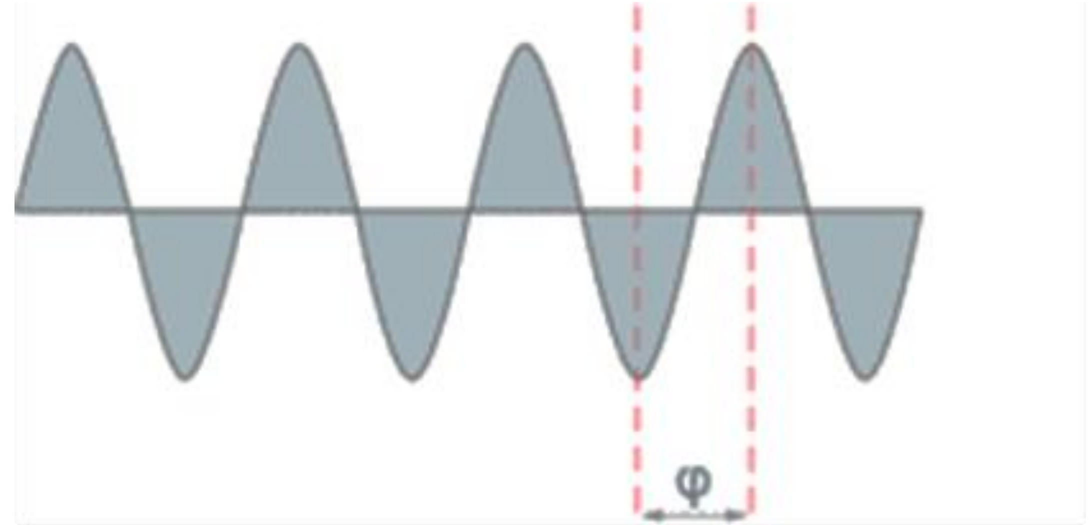


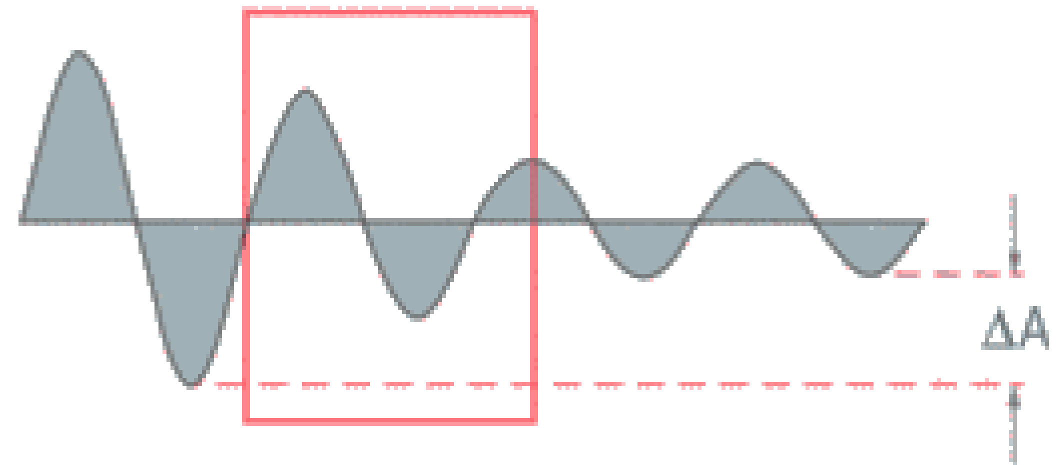
Bright-field light micrograph of a hematoxylin-stained section of *Amphiuma* liver containing hepatocytes and pigment cells. Bar  10 µm.

# Bright field



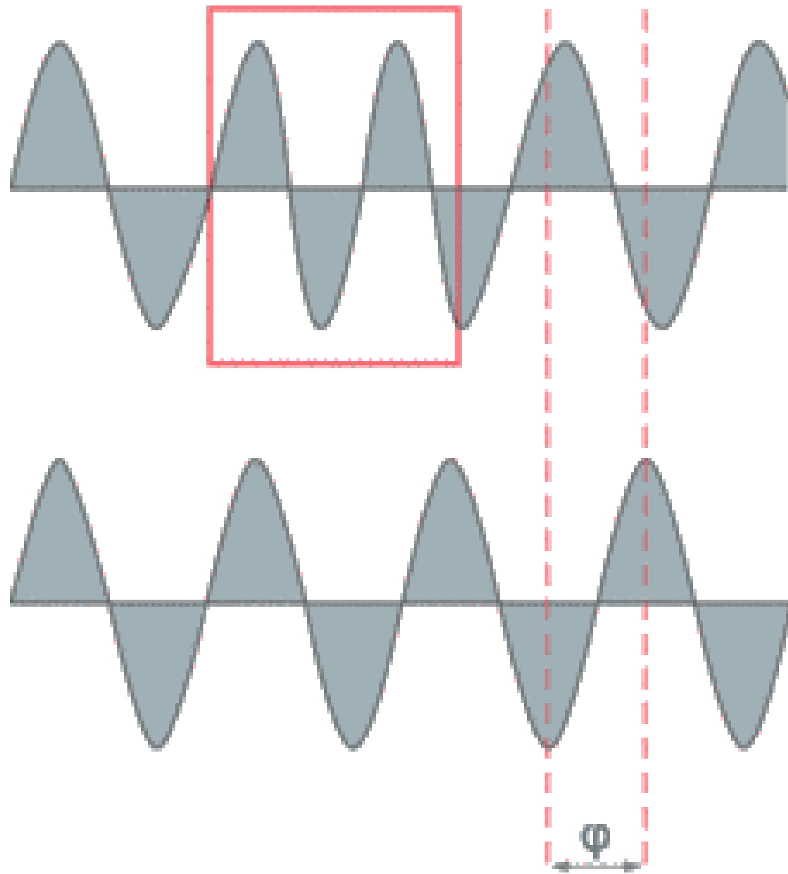
Section taste buds: rabbit

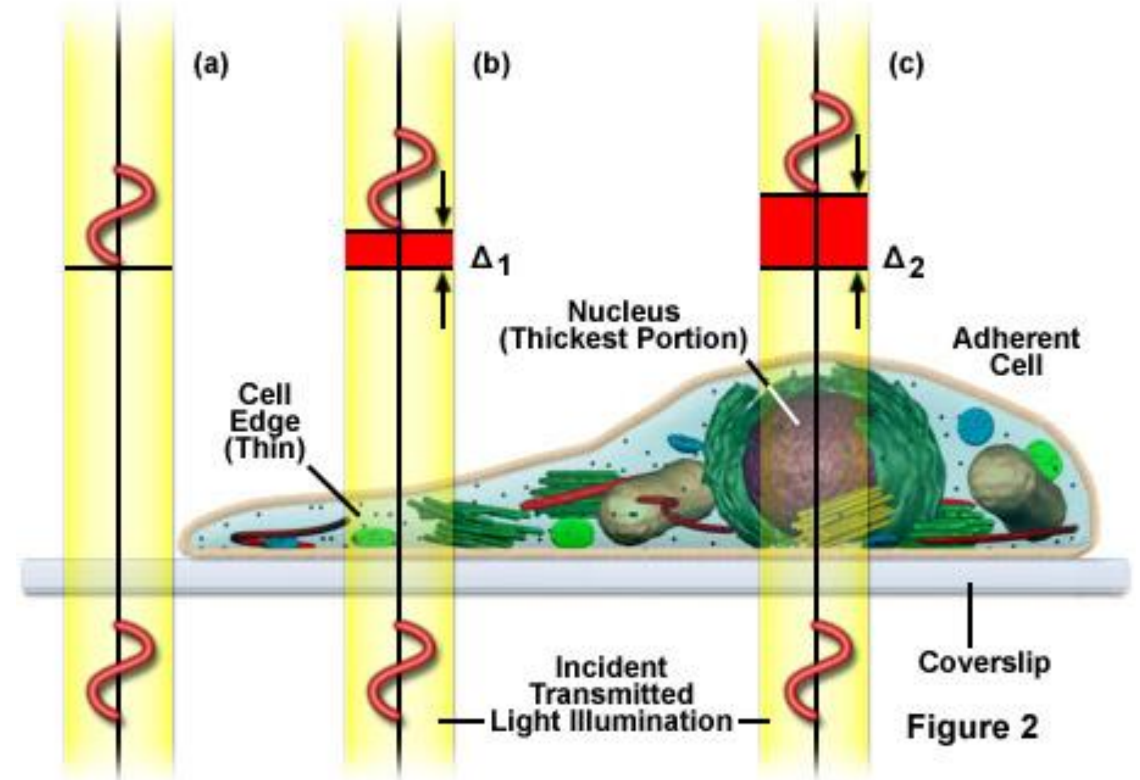



The eye can only experience changes in amplitude (brightness) and in frequency (colour).

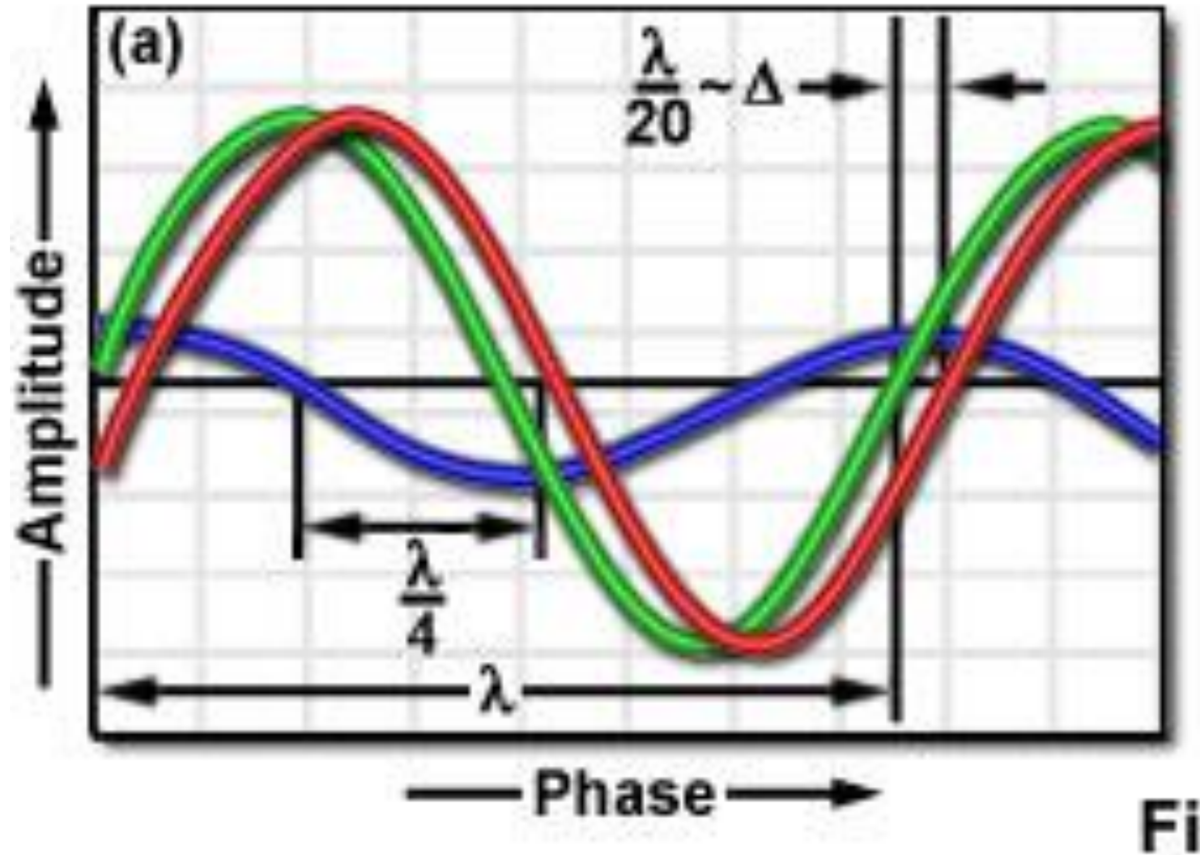
# Phase contrast




Eyes can not detect changes in phase

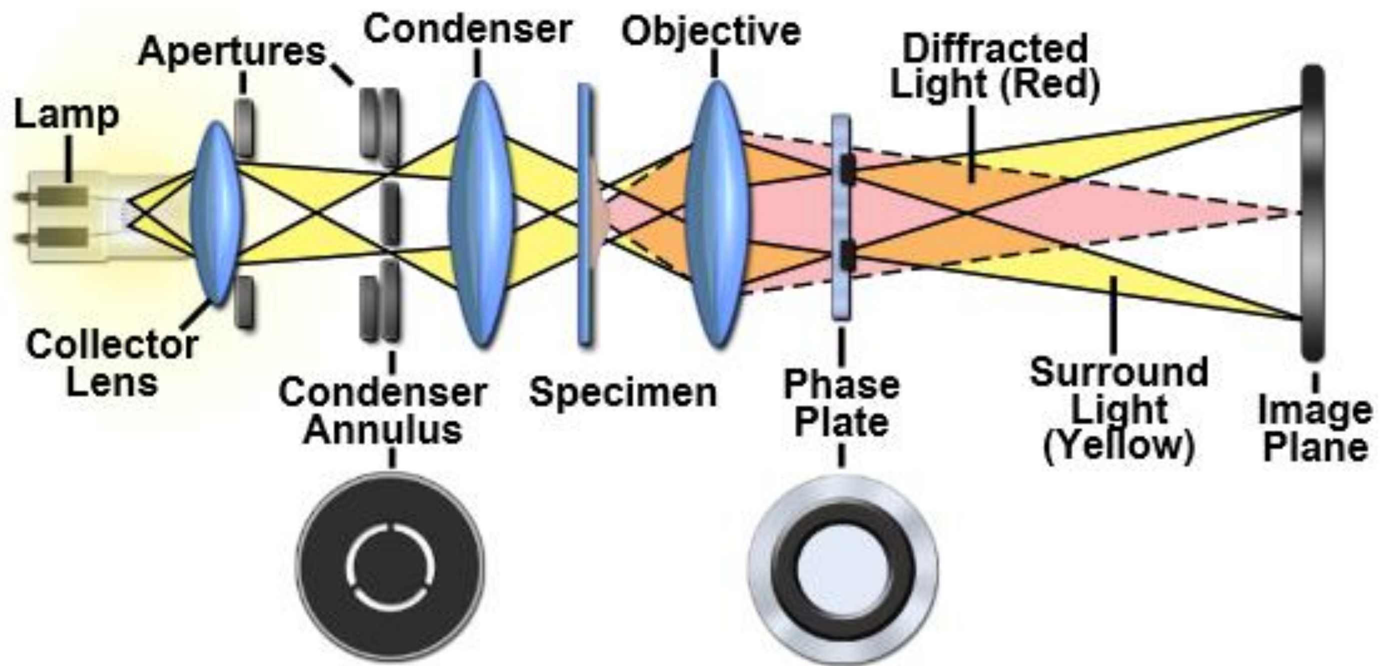
# Phase contrast: In another way

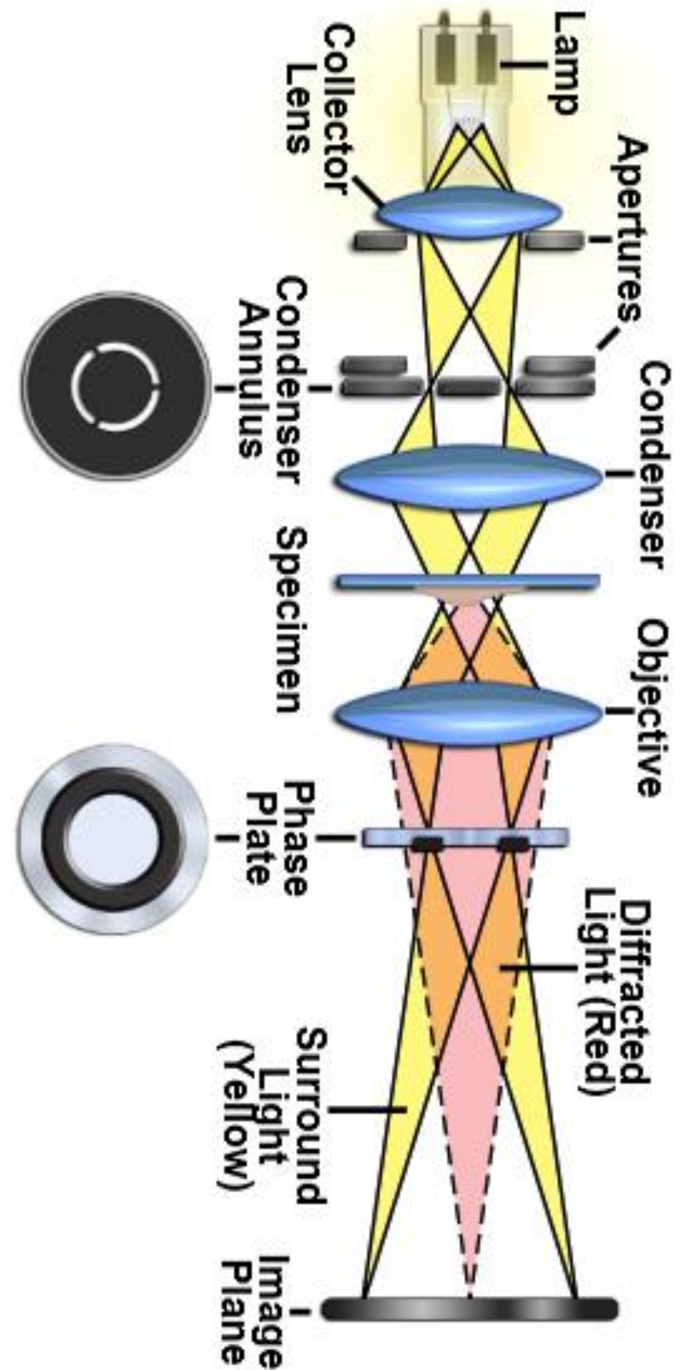



- Let S (red) be light passing through medium surrounding sample and D (blue) light interacting with specimen. S and D typically interfere to yield P (green), which is what we can usually detect.
- P will be phase shifted compared to S, but our eyes cannot detect phase shifts.
- Phase contrast microscopy effectively converts this phase shift into an intensity difference we can detect

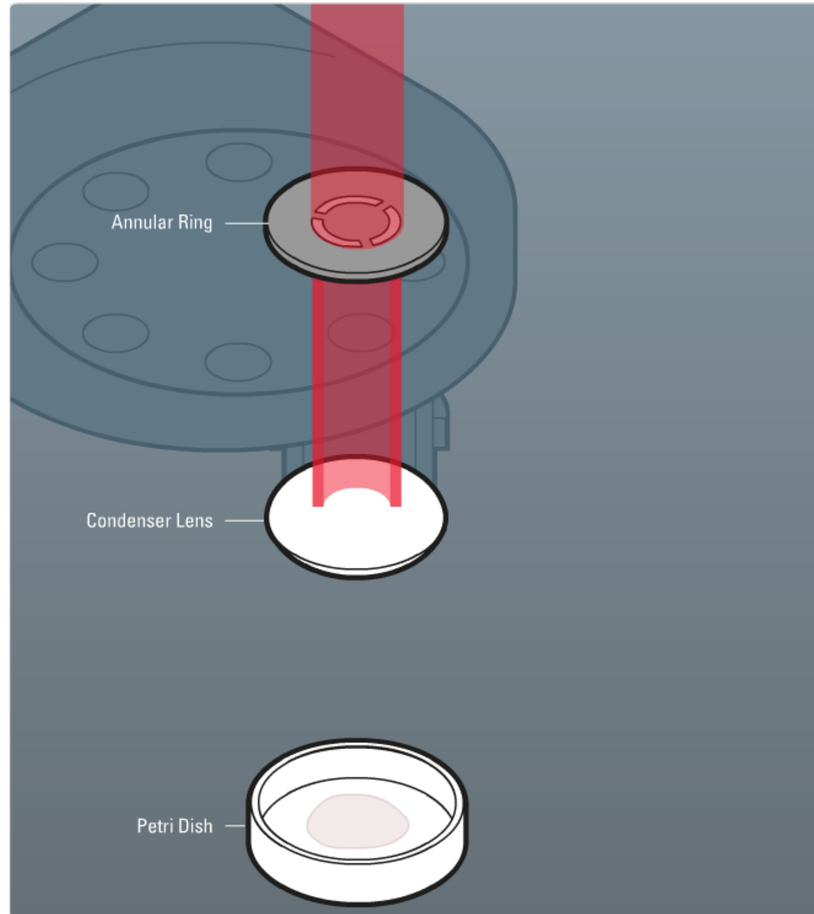
In the 1930s, Frits Zernike, a Dutch physicist at the University of Groningen, created
an optical design that could *transform differences in phase to differences in amplitude.*
1953 Nobel

# Optical path



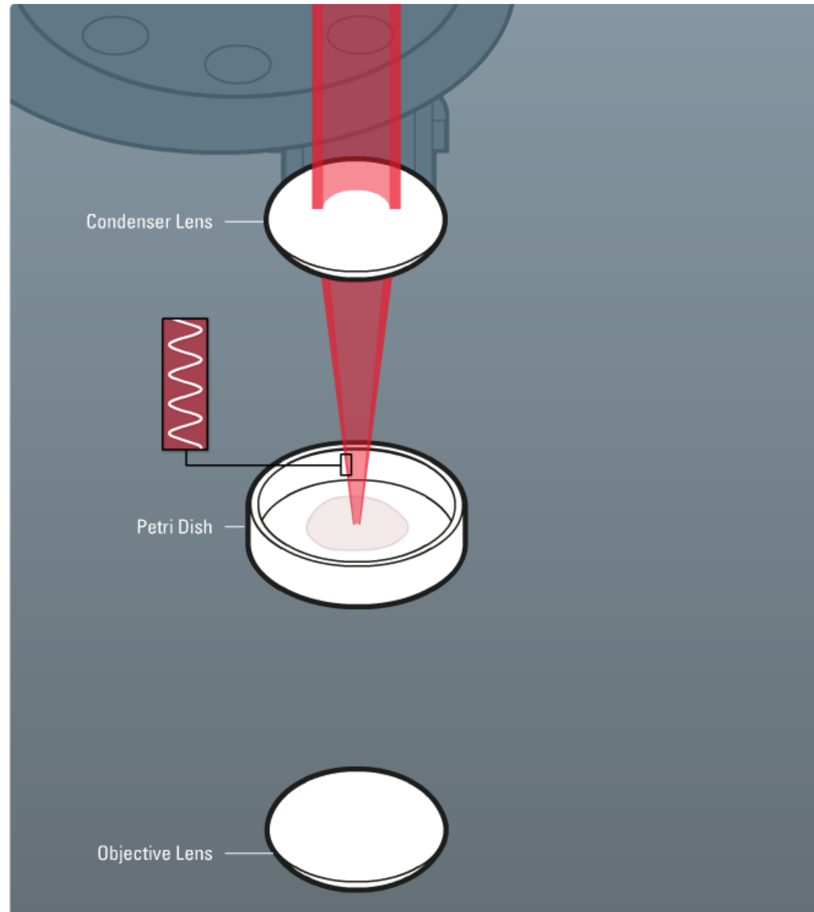



# Step 1



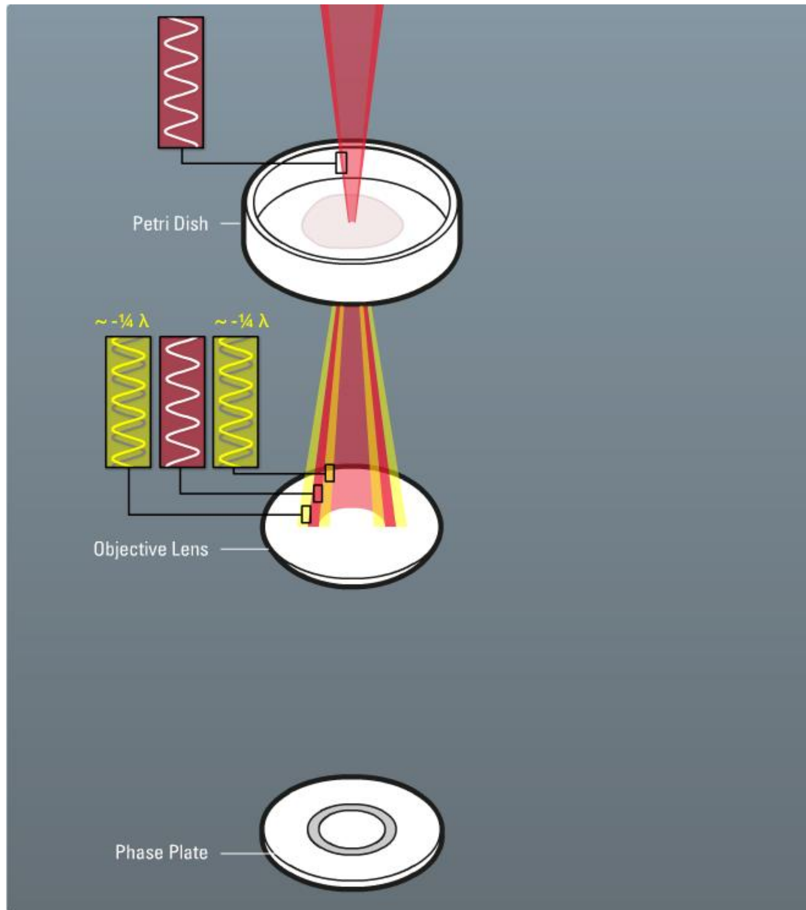

- A very simplified view of the light path
- Light from the source passes through the annular ring and that forms a hollow cylinder onto condenser

# Step 2



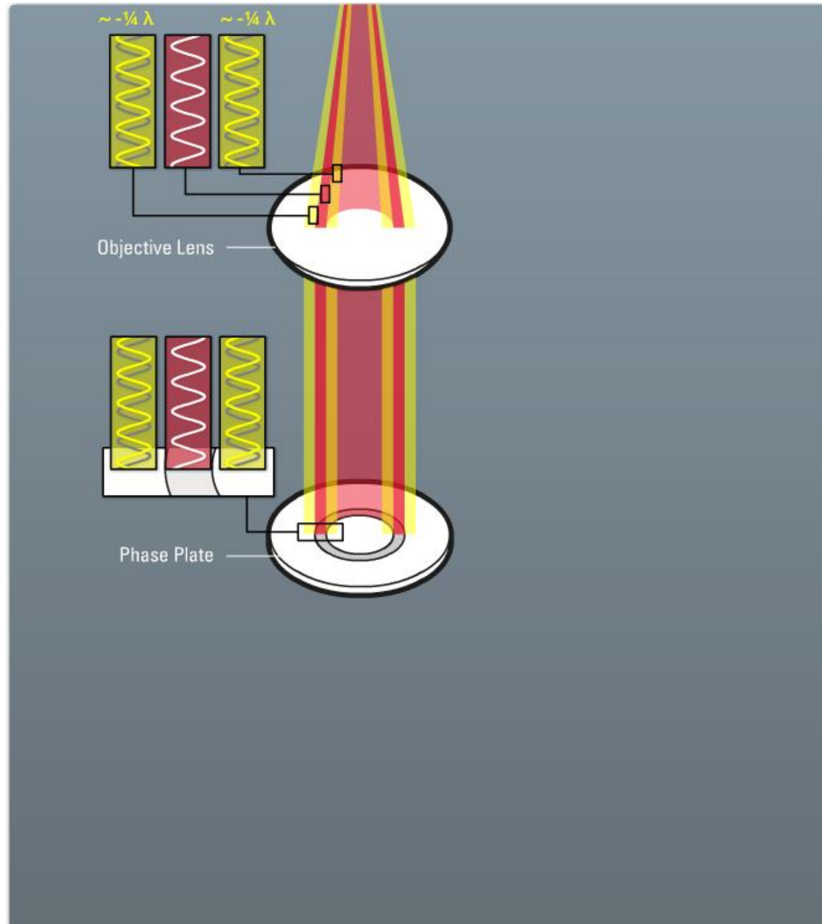

- Condenser focuses the light to specimen
  - Köhler illumination is necessary

# Step 3



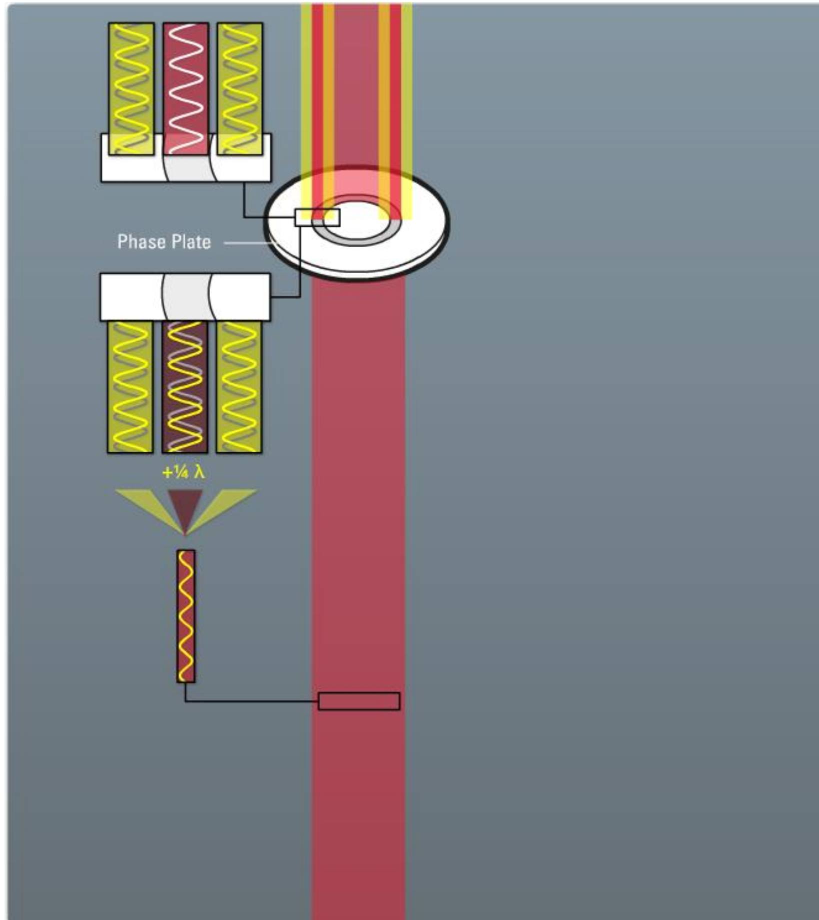

- Deflected light forms the inside and outside boundary of un-deflected light
  - Keep in mind that the yellow light is phase shifted from red
  - Typically the phase shift is $-\frac{1}{4}\lambda$ for biological sample but not always
- The light comes out as hollow cone onto objective

# Step 4



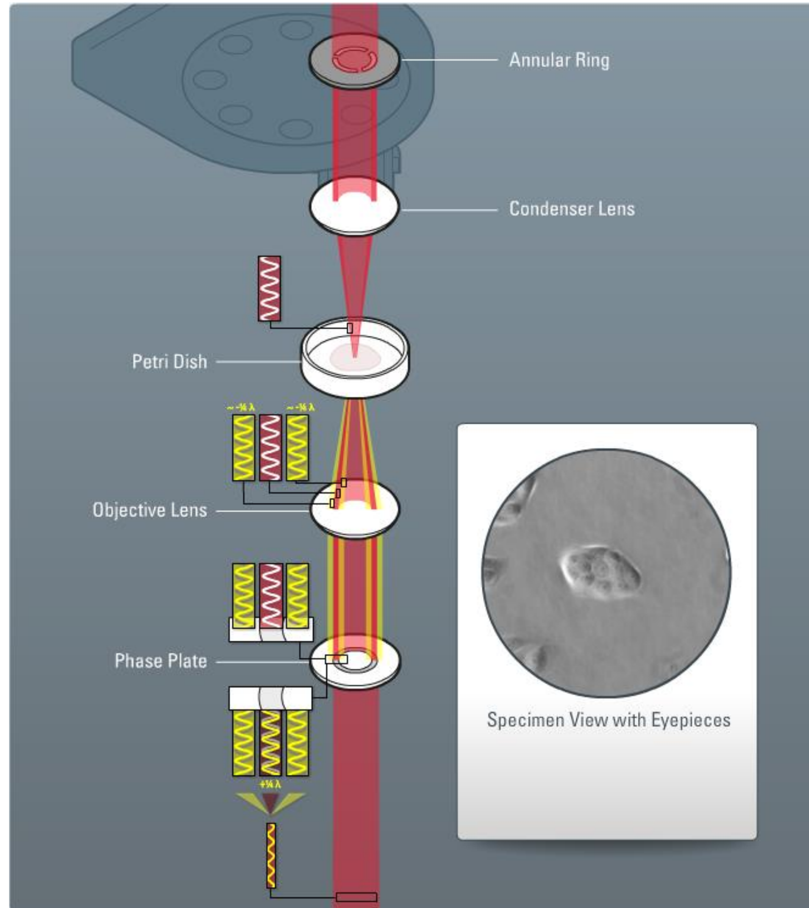

- Objective keeps the phase shifted light spatially separated and passed them onto phase plate
    - Phase plate has to be aligned with annular ring of condenser

# Step 5




- Phase plate is semi opaque and has special coating
- Due to alignment the light that was not deflected hits the phase plate
- Phase plate changes the un-deflected light phase to about +¼λ
- Light that was deflected by the specimen do not change
- That makes difference between deflected and un-deflected light phase difference ½λ
- All the lights are then combined leading to interference that in turn makes phase shifts visible

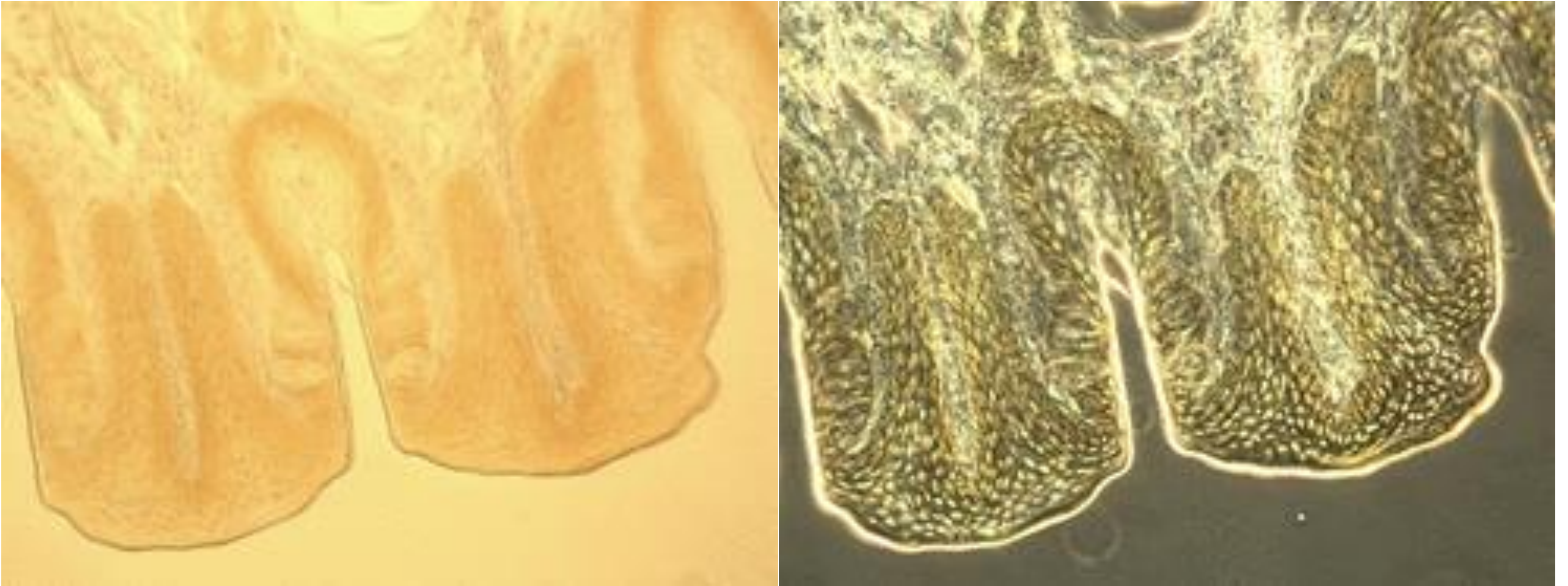
# Step 6




- Total overview again and resulting image

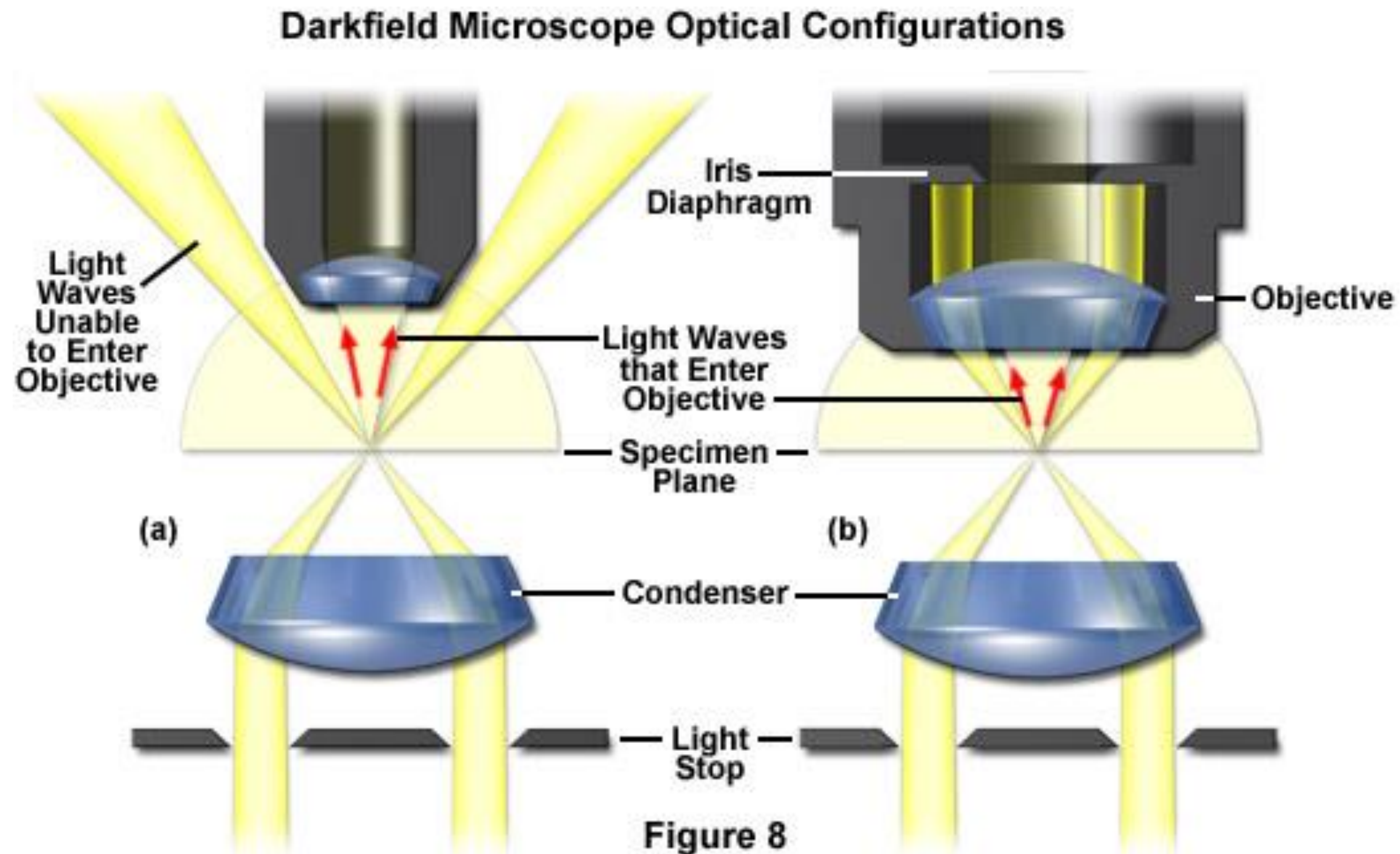
http://www.leica-microsystems.com/science-lab/the-principles-of-phase-contrast/

# End result

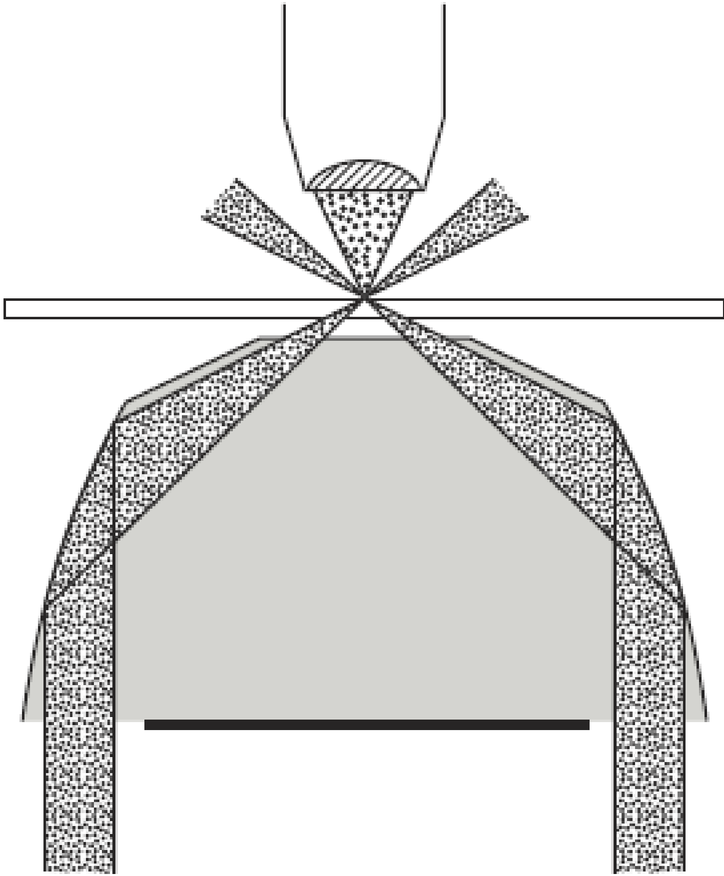


Section taste buds: rabbit

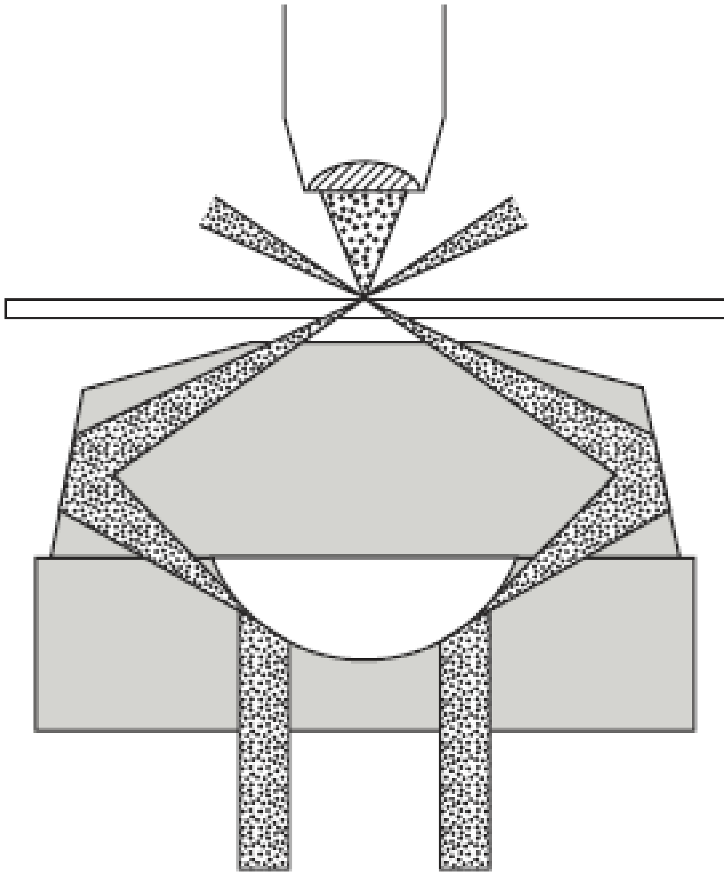
# Dark field microscopy



# Condensors



Paraboloidal

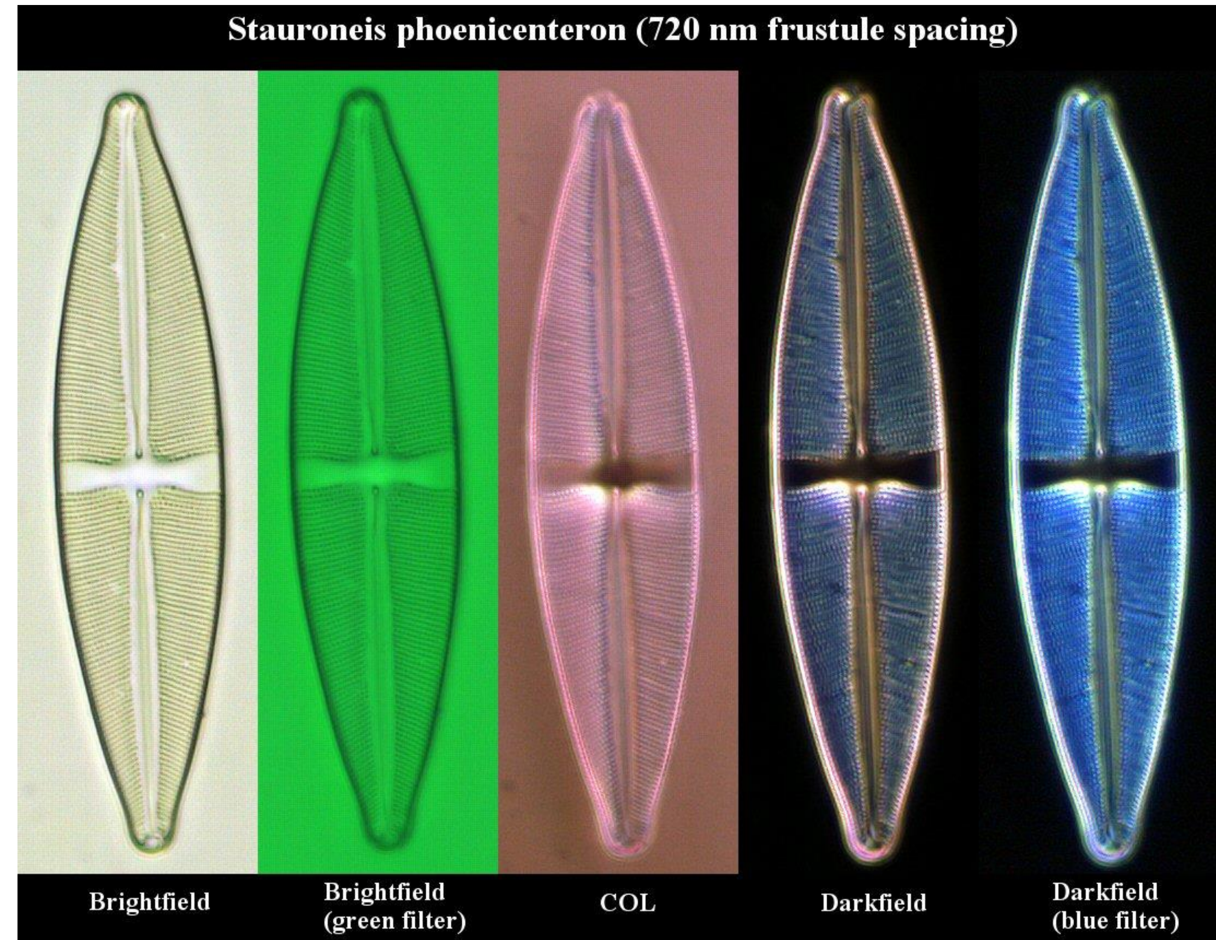


Cardioid

# Theory

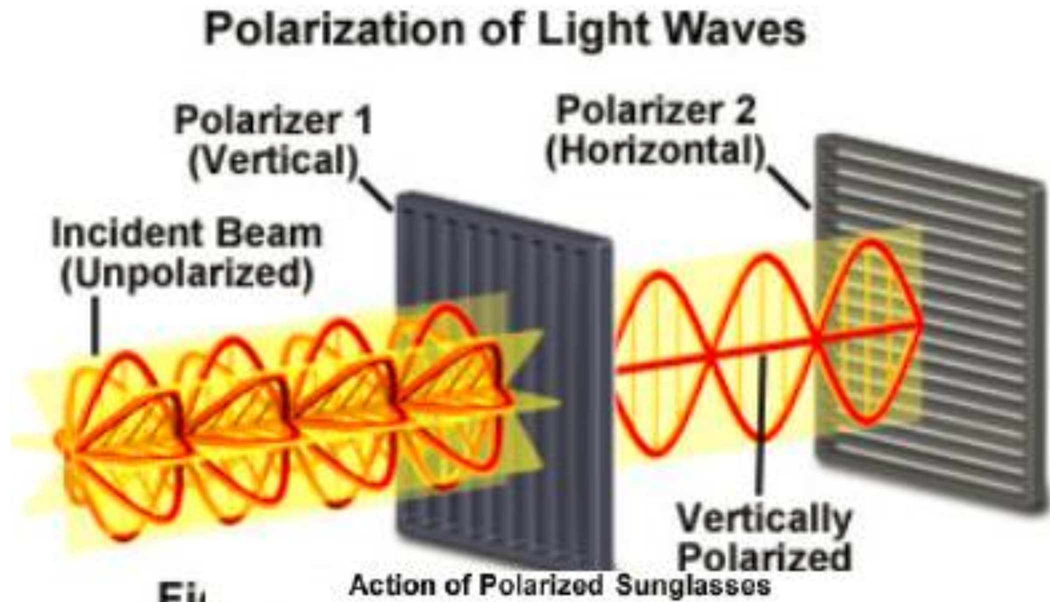
- Dark-field conditions are obtained by illuminating the specimen at an oblique angle such that direct, non-diffracted rays are not collected by the objective lens. Only diffracted light from the specimen is captured by the objective, and the direct waves pass uncollected off to one side of the lens.
- Much better images are obtained with a special dark-field condenser annulus, which is mounted in the condenser turret. Special oil immersion dark-field condensers must be used for oil immersion objectives.
- Dark-field microscopy resembles phase contrast microscopy in that the specimen is illuminated by rays originating at a transparent annulus in the condenser.
- However, in dark-field optics only diffracted rays are collected by the objective and contribute to the image; nondiffracted rays are pitched too steeply and do not enter the lens. Since nondiffracted background light is absent from the image, light-diffracting objects look bright against a dark field.

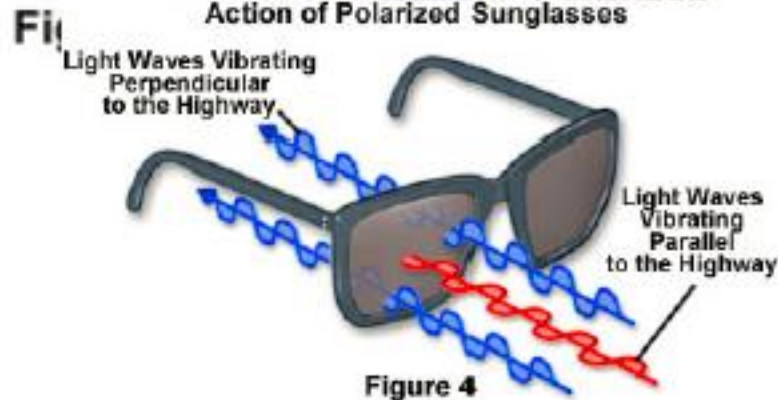
# End result



# Differential Interference Contrast (DIC) Microscopy

First: Polarity



Bright field

Phase contrast

Nomarski differential interference

Darkfield

# Study materials

**Online**

- http://zeiss-campus.fsu.edu/index.html
- https://www.leica-microsystems.com/science-lab/
- https://www.microscopyu.com/
- http://www.olympusmicro.com/

**Books**

- Wilson, Walker: Principles and Techniques of Biochemistry and Molecular Biology (7th Ed)
- D. B. Murphy: Fundamentals of Light Microscopy and Electronic Imaging (1st Edition)
  - Only read this if you are really interested in microscopy