

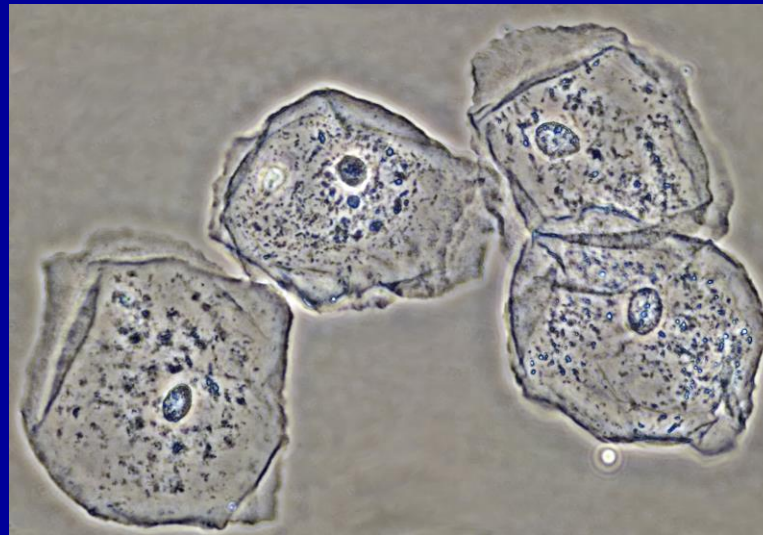
# Manchester Microscopical & Natural History Society

*Established 1880*

*[www.manchestermicroscopical.org.uk](http://www.manchestermicroscopical.org.uk)*



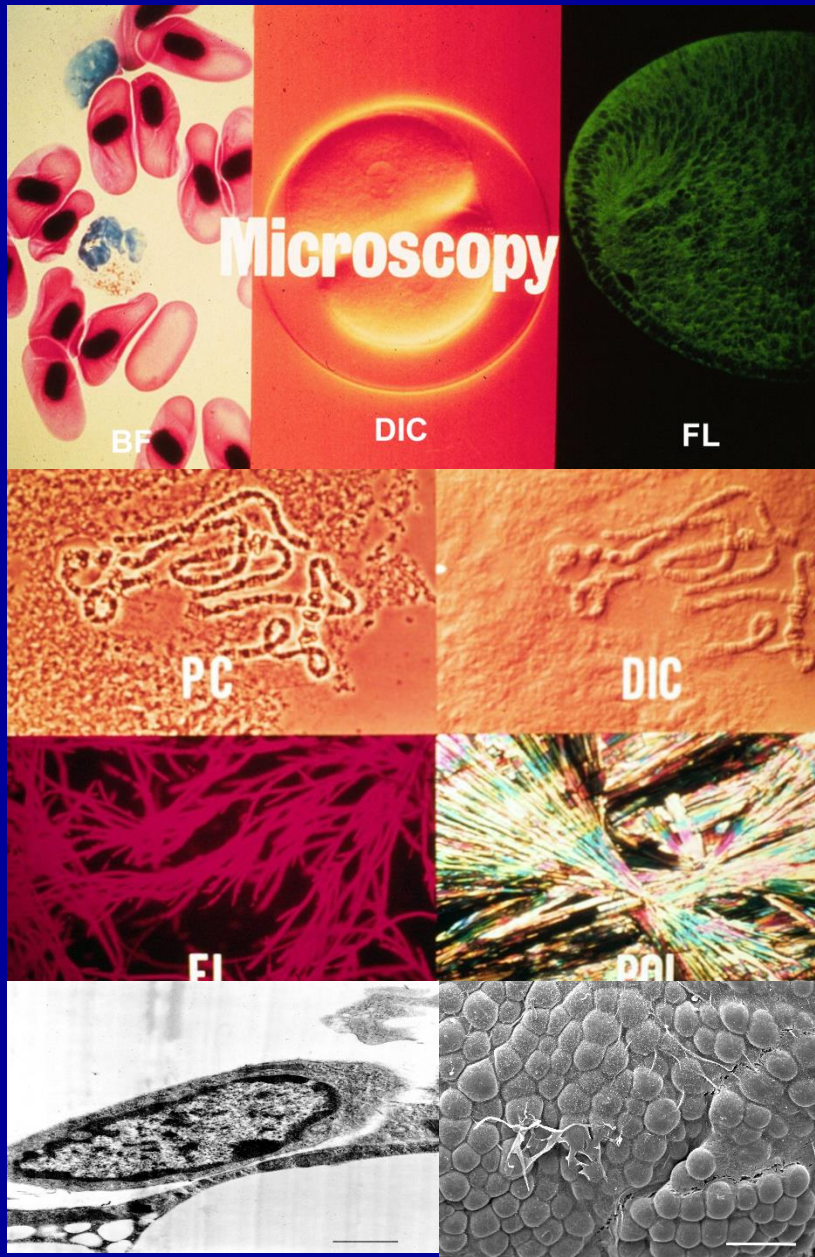
## Phase-Contrast Microscopy



**Talk &  
Practical**

*Mike Mahon, November 23<sup>rd</sup>, 2024*

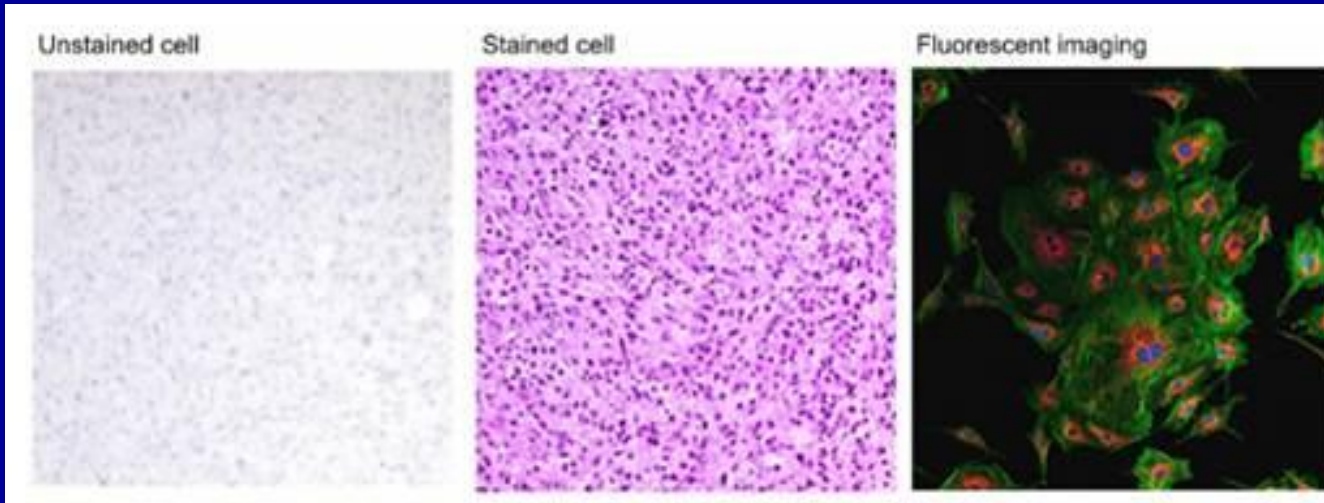
# Which technique to use - & how ?



- Light Microscopy
  - bright field / reflectance
  - dark field
  - polarising
  - **phase contrast**
  - interference
  - fluorescence
  - computerised / confocal
- Electron Microscopy
  - Transmission +
  - Scanning +
- Scanning Probe/Stylus

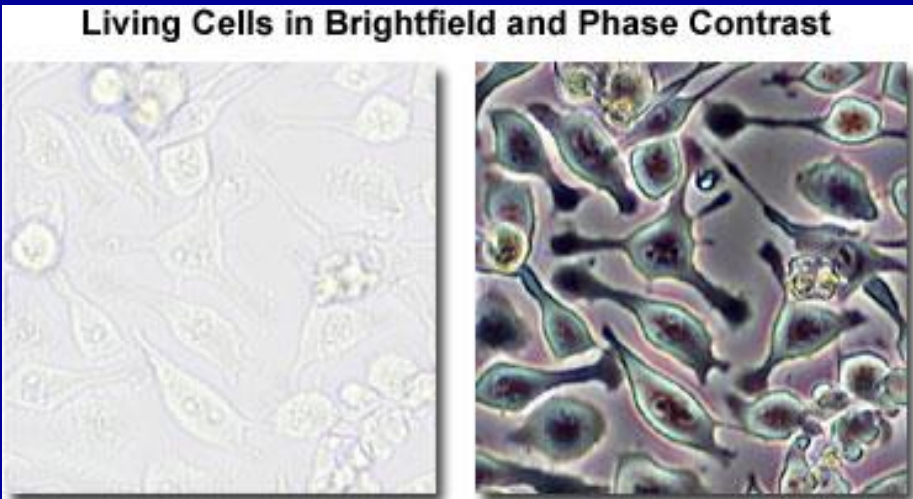
# The Problem ?

Unstained biological cells and tissue slices are hardly visible under the microscope.



Add contrast by –  
killing  
fixing  
embedding  
staining  
labelling

--ve Not natural !



Or view directly by **Phase Contrast**

++ Living, dividing, moving, ...

**Add Contrast Optically**



# Microscopy Landmarks

- <1600s magnifying lens
- 1600s simple microscopes
- 1650s compound microscopes
- 1830s expansion of light microscopy + pol, DF
- 1900-80 UV & fluorescence microscopy
- **1930-1950s phase contrast & interference**
- 1930-60 electron microscopes developed
- 1980s confocal microscopy
- 1990s-2010s scanning probe microscopies
- 2010s super-res,  $\mu$ CT, X-ray, 3D, AI microscopies,...

# History



Frits Zernike  
(1888-1966)



- 1930s Theory/Invention  
Diffraction gratings > Microscopes
- 1942 Zeiss
- 1953 Nobel Prize

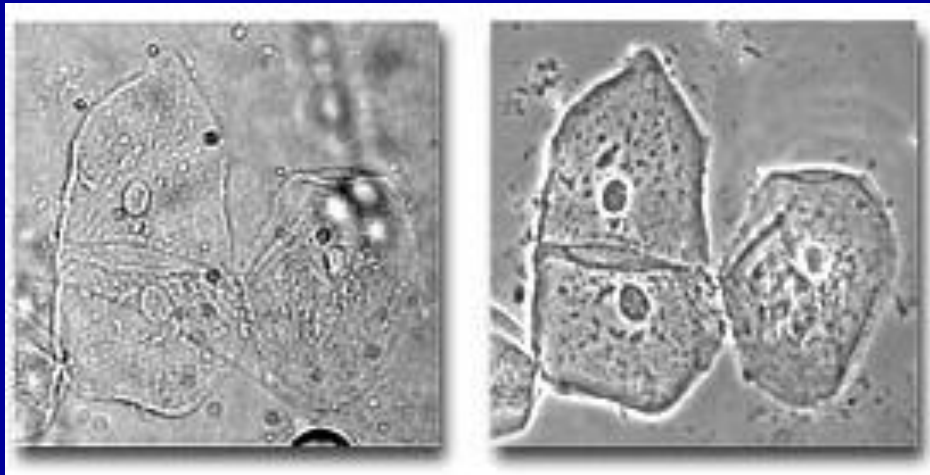
FRIJTS ZERNIKE  
How I discovered phase contrast  
Nobel Lecture, October 11, 1953

These contrast cases are discussed while working with microscopes, but as a different part of optics I turned from my interest in diffraction gratings, about 1920 on. Such a reflecting grating consists of glass or quartz plates with a large number of equidistant grooves etched on its surface. That means considerable requirements as to the homogeneity of the grooves themselves in the optical behavior of the grating. The most conspicuous error is a periodic one which repeats itself after each wavelength of the source of the reflecting angles. The regularly occurring displacement of the grooves causes corresponding changes of the optical path, and so the mirror surface was wavy. Consequently the instrument behaves as if a convex grating, with a constant of about half a millimeter, were superimposed on it in each working spectral line incorporated in light and left by a number of weak spectral lines, the visible spectrum of light.

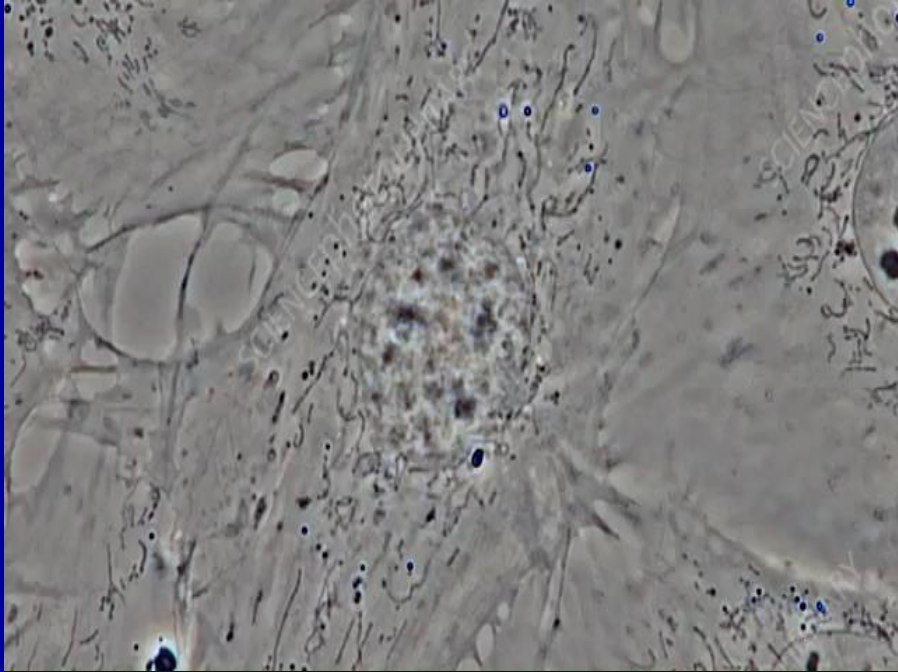
There was a second effect of some kind, which is of the order of the grating, giving the ray an additional optical path. A similar grating, used in the microscope, is usually discussed as a surface, but I thought of the optical path. It finally occurred to me to use a strongly etched surface. At the end of 1932 paper 11, 5, I then constructed the first phase-contrast grating and, but simply the effect of the combination between the principal lens and the grating, indeed the rings disappear when the grating is covered by 1/2 wavelength strongly absorbing film (inclusion of a cavity). On the contrary I was convinced that the etched surface gave more information about the periods, being even than that obtainable by photographing the grating, because in the line case the relative phase of the grating surface plus phase-contrast surface is the same in each case. Thus the question is void, proving to look further on it as soon as an opportunity would arise.

About 1933 our laboratory had obtained a large concave grating and we tried to design a microscope. The original appearance of the resolution was very hard, but as the grating was not better than the one I had a small laboratory printed of the grating. Then the unexpected happened. The contrast was very clear, but disappeared as the microscope was nearly focused.

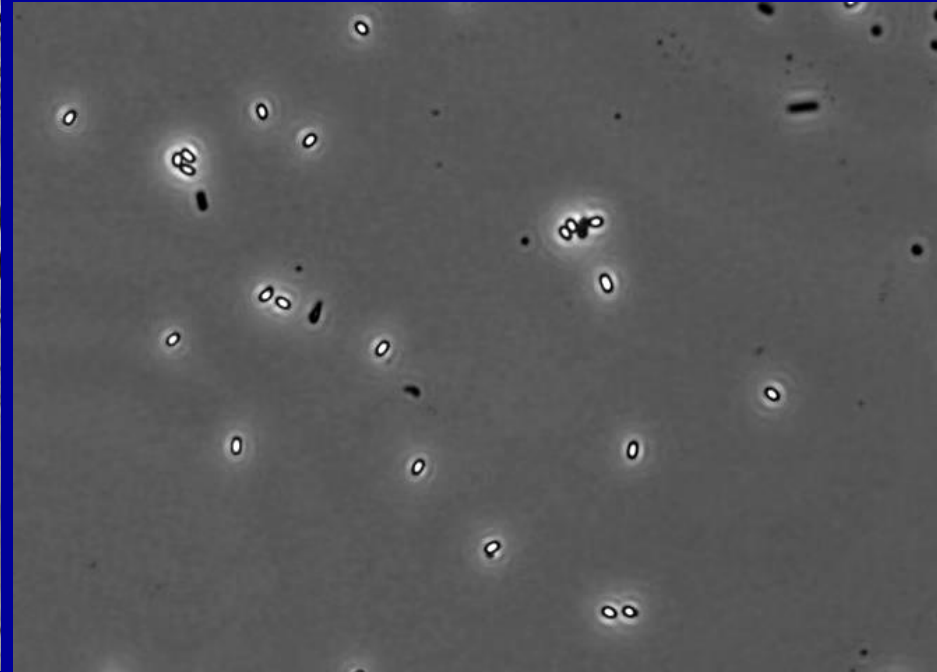
< Click image to view on web



# Videos



[Cell division, timelapse light microscopy - Stock Video Clip - K005/7812 - Science Photo Library](#)



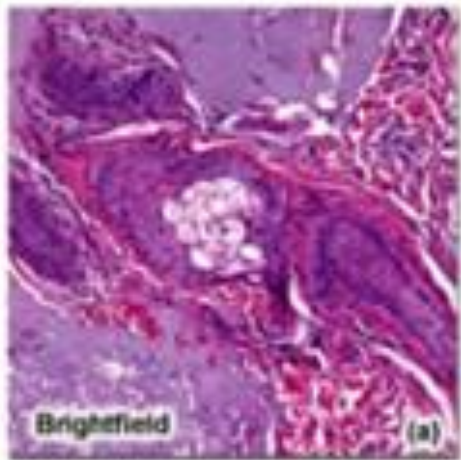
[File:Live-Cell-Imaging-of-Germination-and-Outgrowth-of-Individual-Bacillus-subtilis-Spores-the-Effect-of-pone.0058972.s005.ogv - Wikimedia Commons](#)

[Historic time lapse movie by Dr. Kurt Michel, Carl Zeiss Jena \(ca. 1943\)](#)

Area Light: Spread

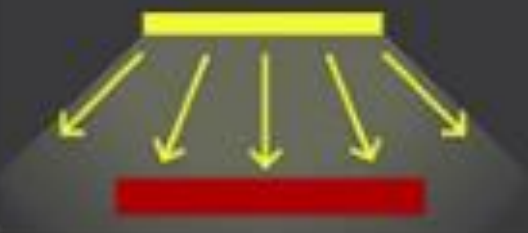


Brightfield microscopy

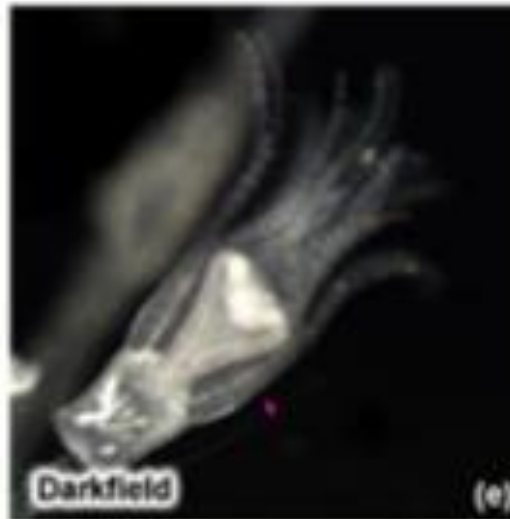


Staining needed

Area Light: Spread

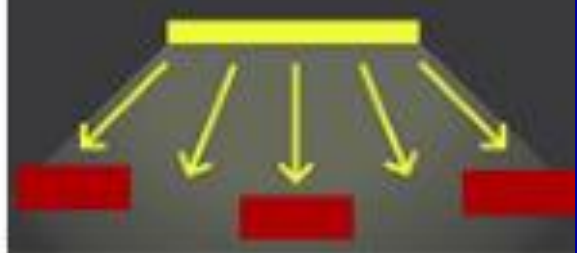


Dark Field microscopy

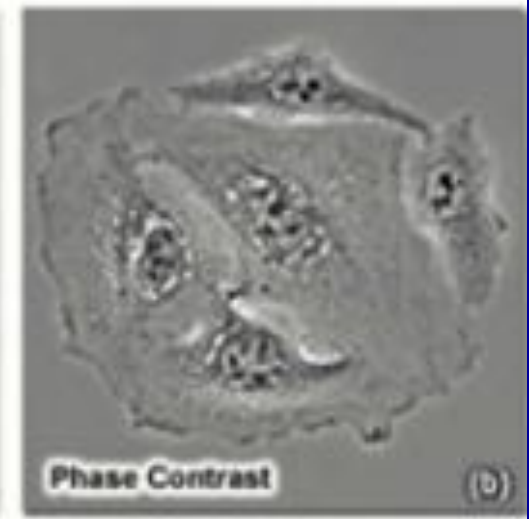


No staining needed

Area Light: Spread



+++  
Phase Contrast Microscopy

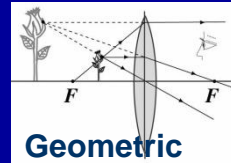


No staining needed

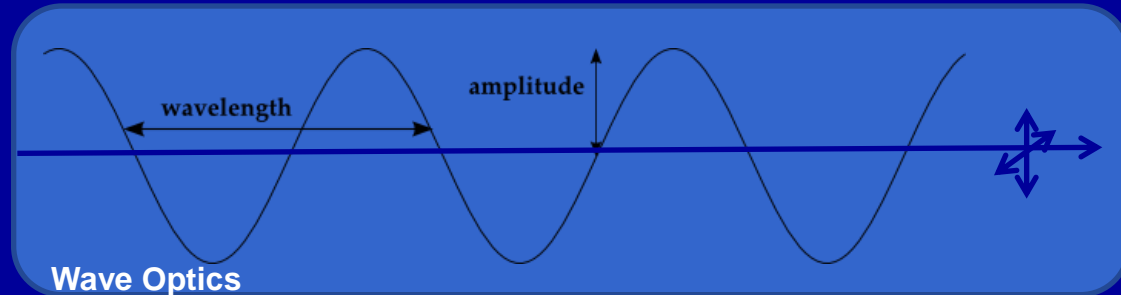
# Light Microscope Theory

## Properties of Light

- Geometric Optics
  - image location
  - Magnification / Res
- Wave Optics
  - direction
  - amplitude
  - wavelength
  - phase
  - vibrational plane
  - velocity
- Quantum Optics
  - formation & destruction
  - fluorescence



## Microscopies



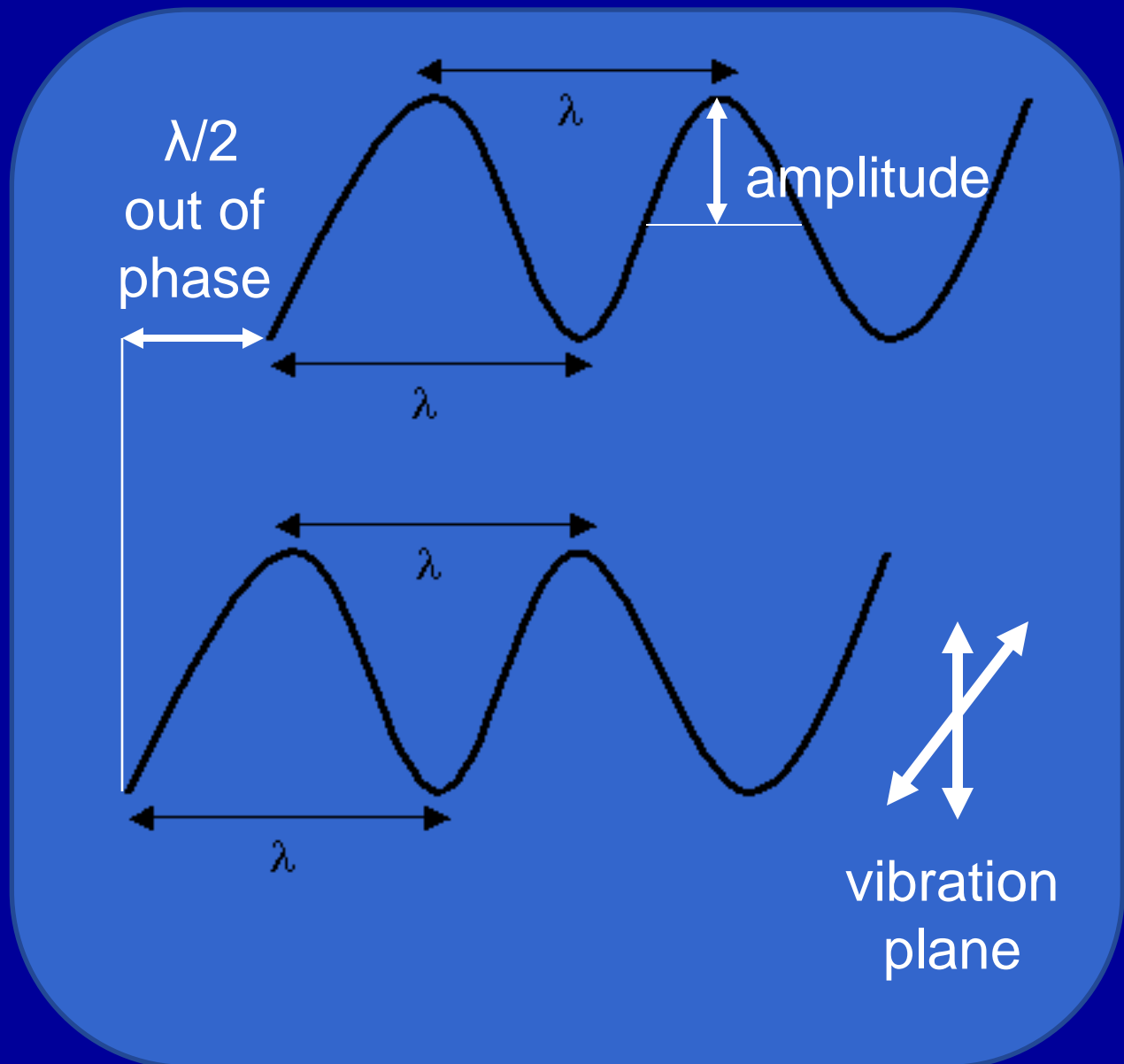
- reflection, refraction, diffraction / BF / DF
- absorption / BF
- colour
- phase contrast, interference
- polarisation
- refraction / oil immersion





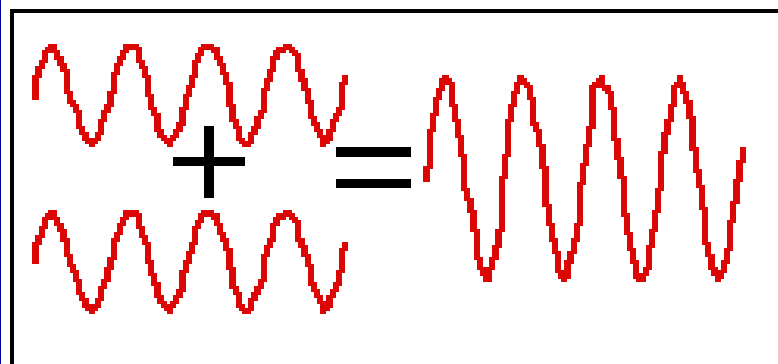
# Properties of Light

- amplitude
- vibration plane
- phase



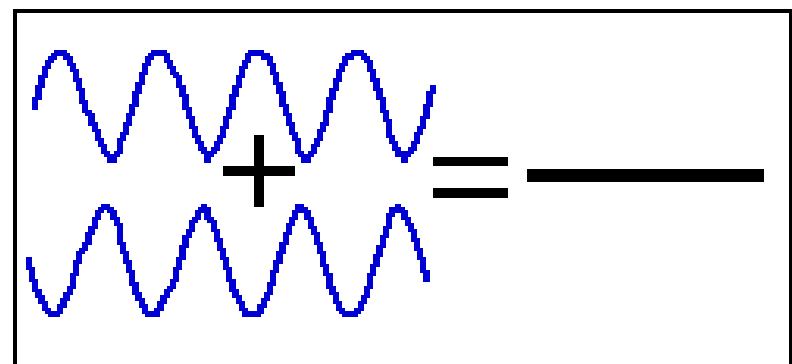
# Phase contrast microscope 2

In Phase



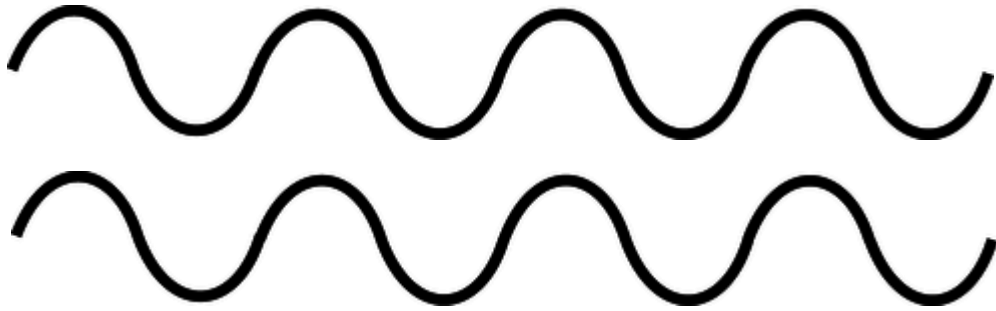
Constructive Interference

Out of Phase

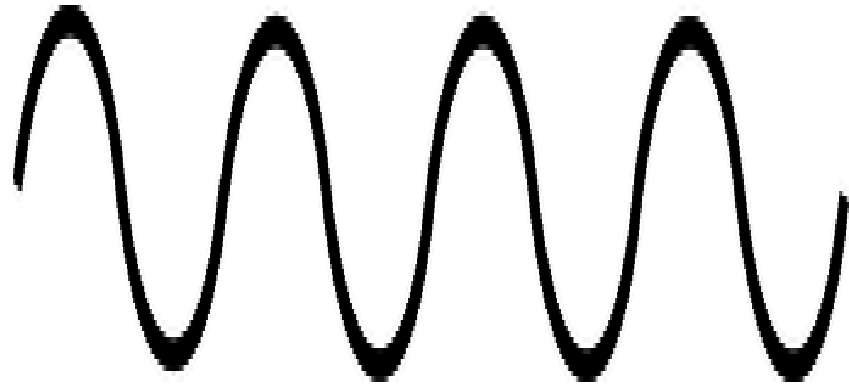


Destructive Interference

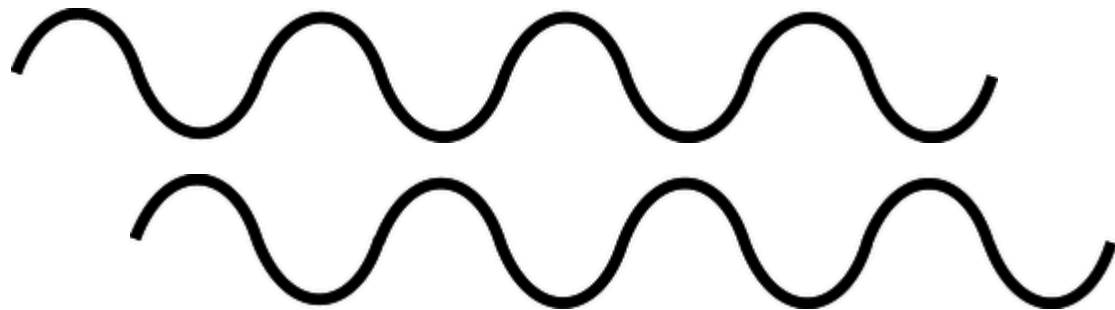
- Waves in phase reinforce each other
- Waves  $\lambda/2$  out of phase destructively interfere



Waves in phase



**Constructive  
Interference**



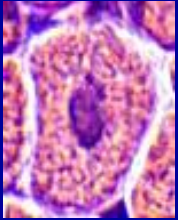
Waves out of phase by  $\frac{1}{2} \lambda$  ( $180^\circ$ )

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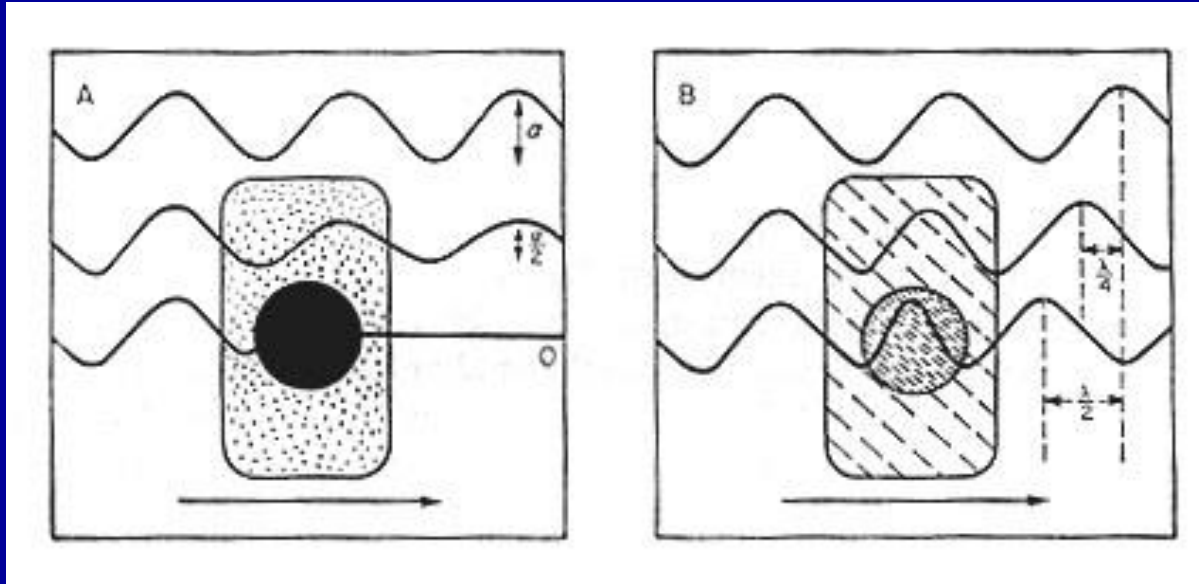
**Destructive  
Interference**

Waves out of phase by  $\frac{1}{4} \lambda$  ( $90^\circ$ ) = hardly noticeable change in amplitude !

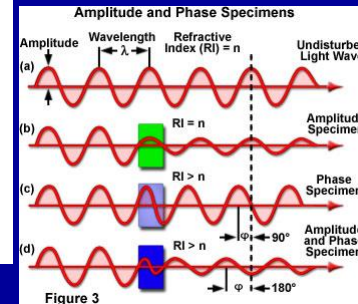
# Brightfield Microscopy: Effect of objects on light waves



stained cell



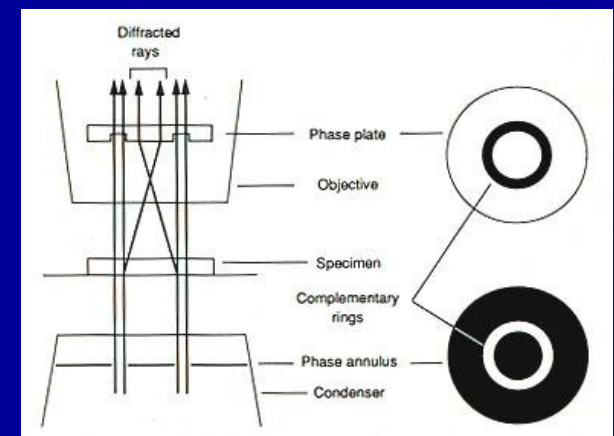
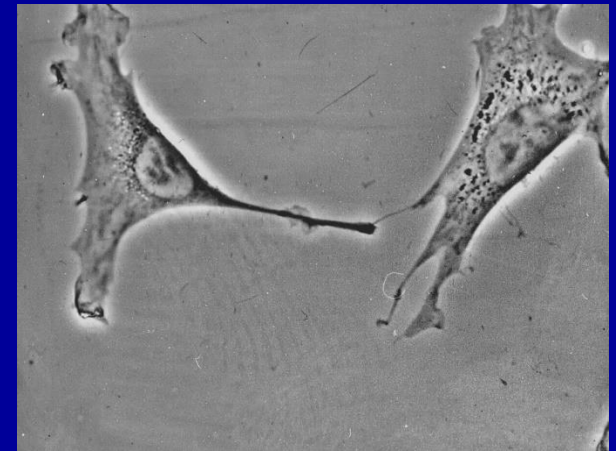
unstained cell



- stained cell reduces amplitude of wave
- unstained cell does not affect amplitude of wave
- unstained cell retards wave due to difference in refractive index
- retardation  $\lambda/4$  for cytoplasm in this case
- (Overall Phase Shift = OPD = RI x Thickness !)

# Phase contrast microscope 1

- invented by Zernike 1935  
developed by Zeiss 1942
- converts differences in phase to differences in amplitude
- simple to set up but requires:
  - phase annulus in condenser
  - phase plate in objective

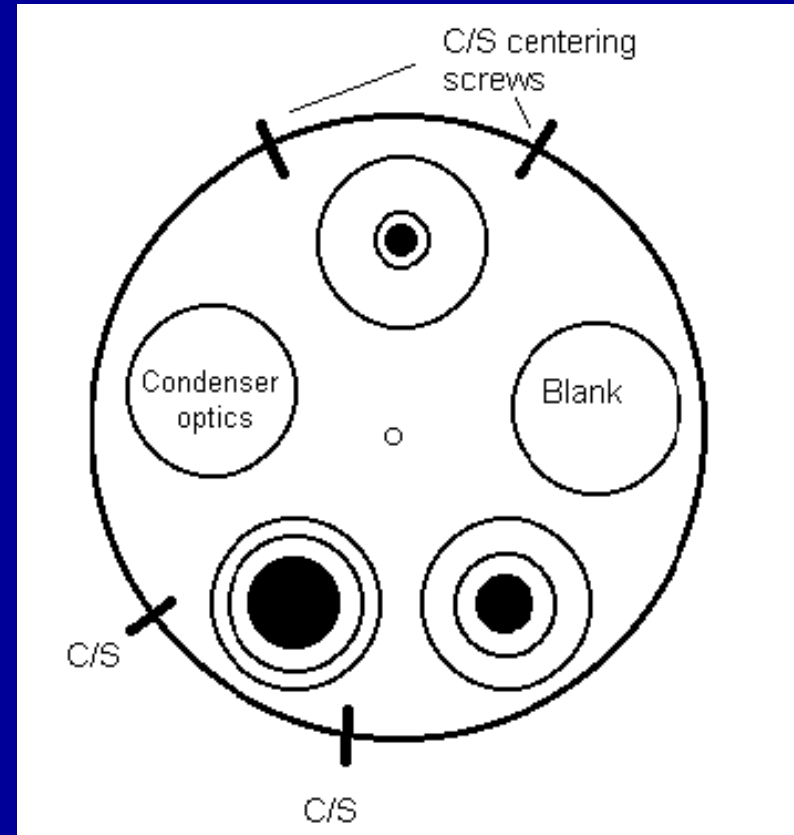




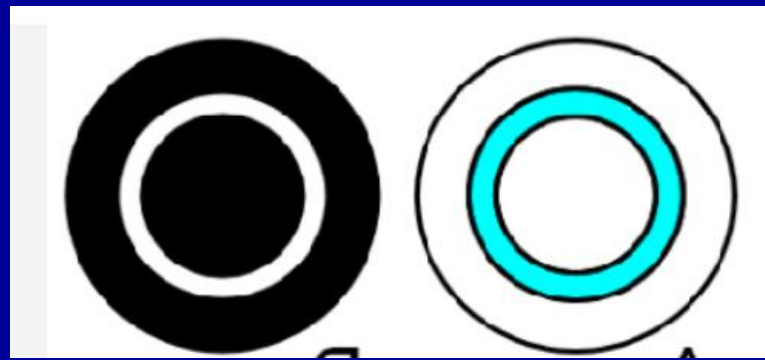
# Phase Condensers



Or ...



# Phase Objectives



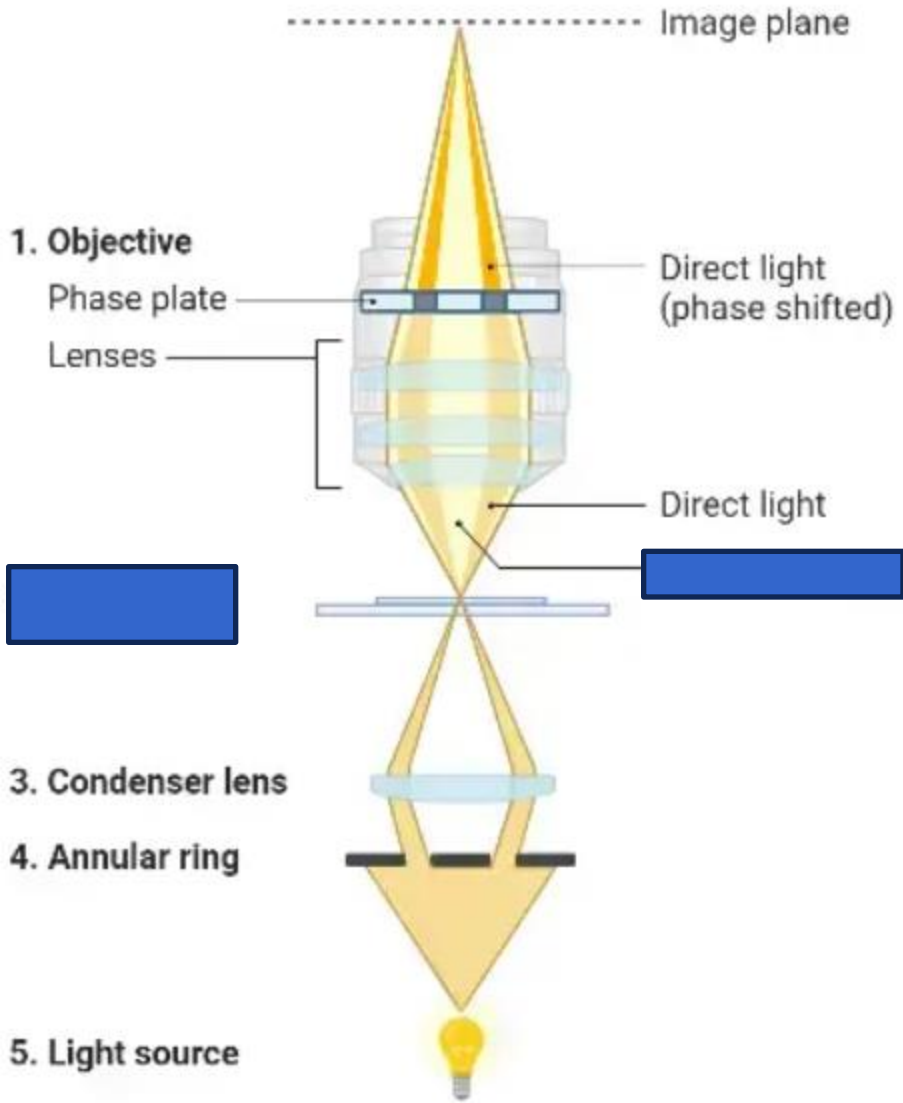
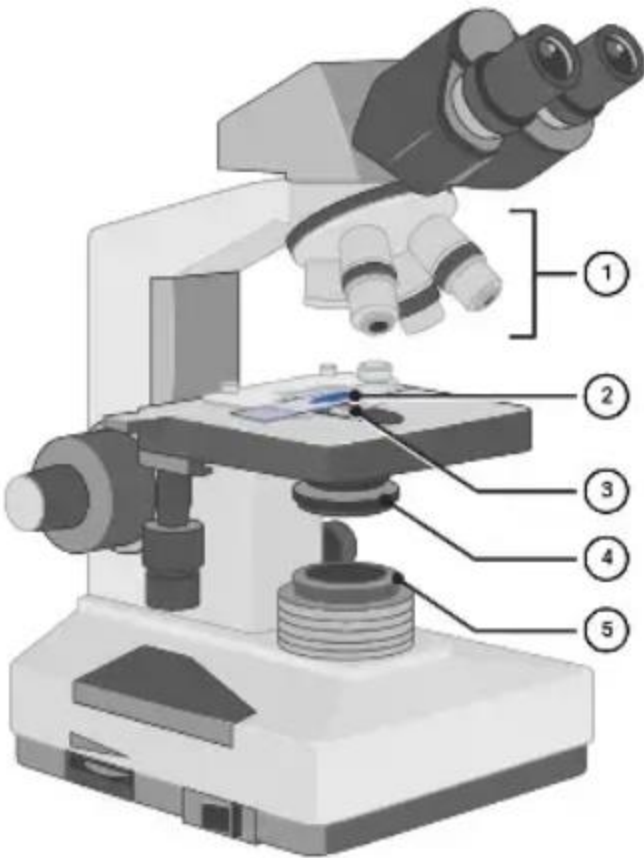
Condenser Annulus

Objective Phase Plate

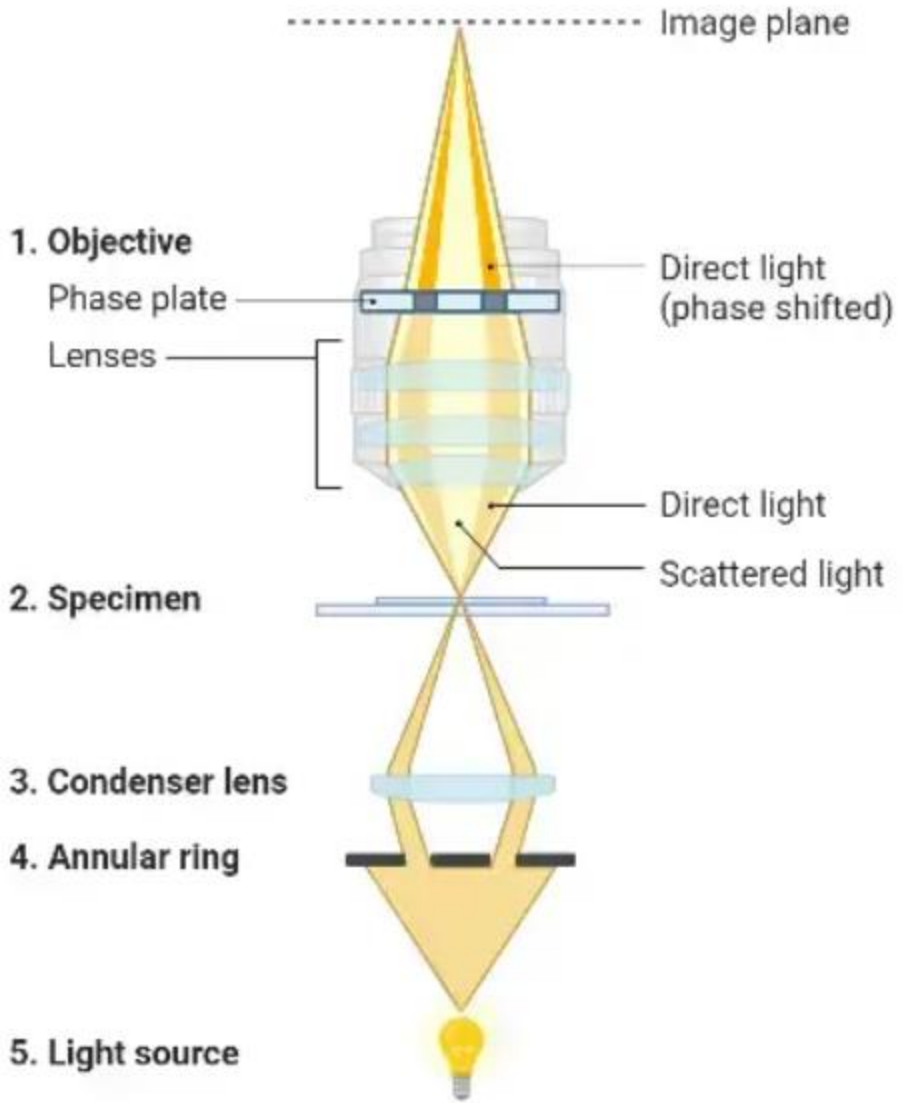
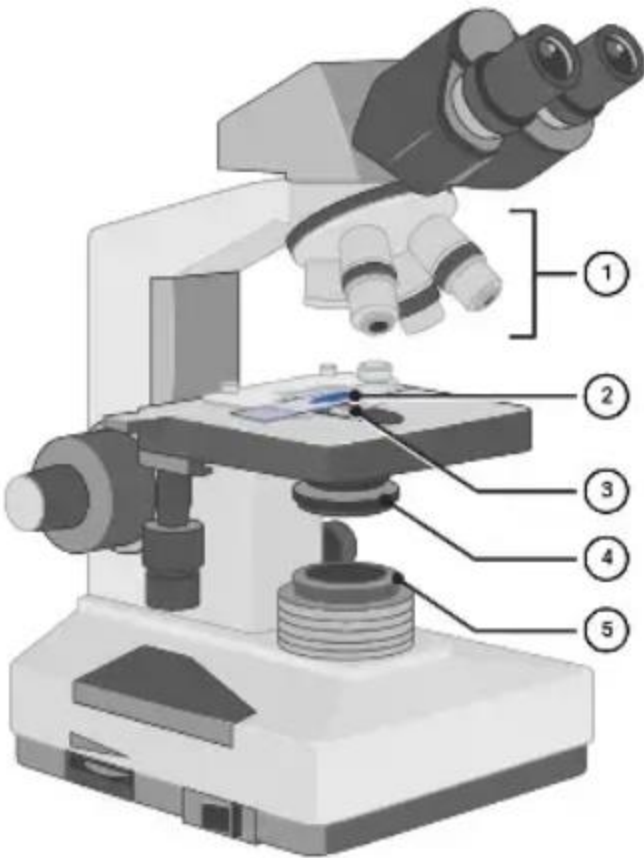




# Phase Contrast Microscopy 2

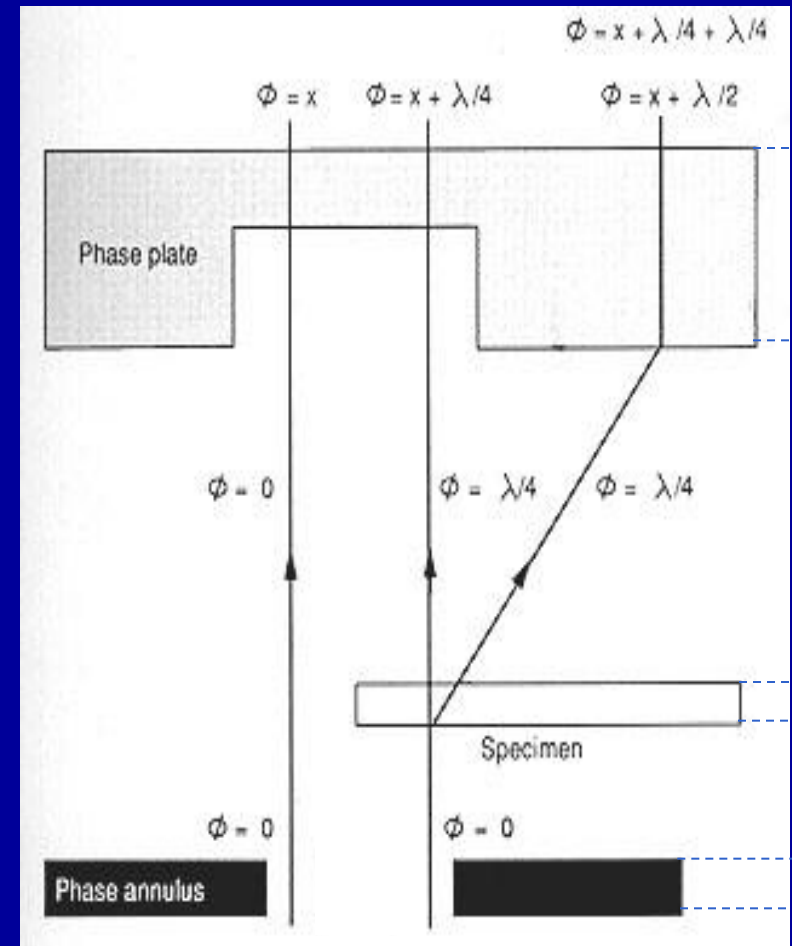


# Phase Contrast Microscopy 2



# Phase contrast microscope 3

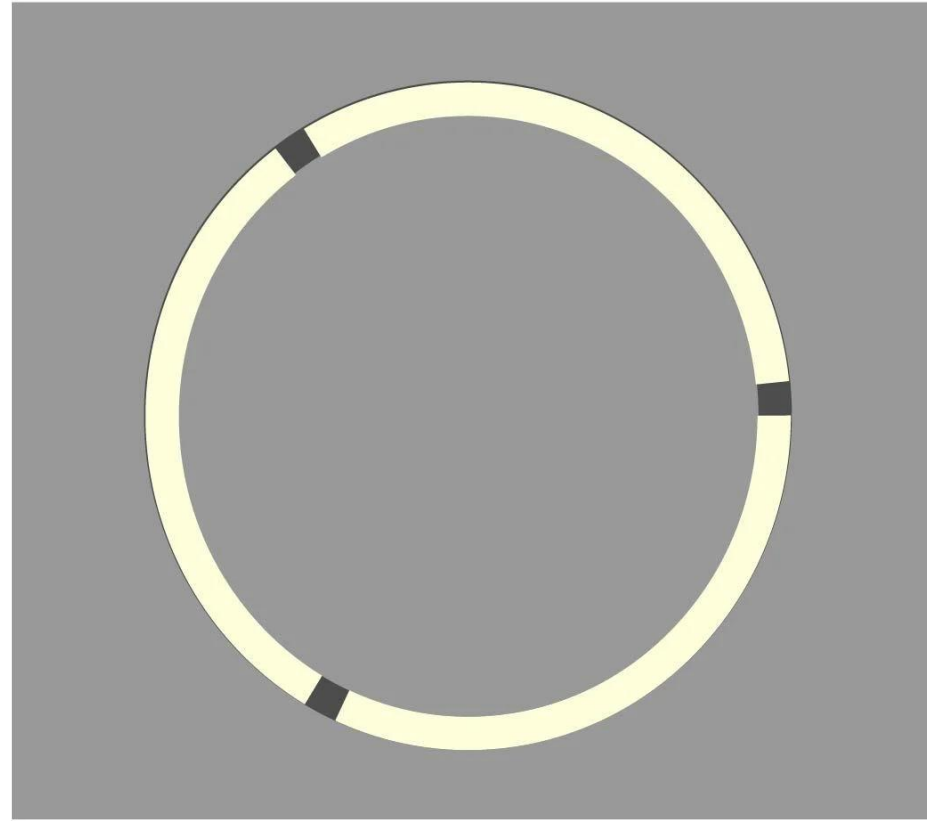
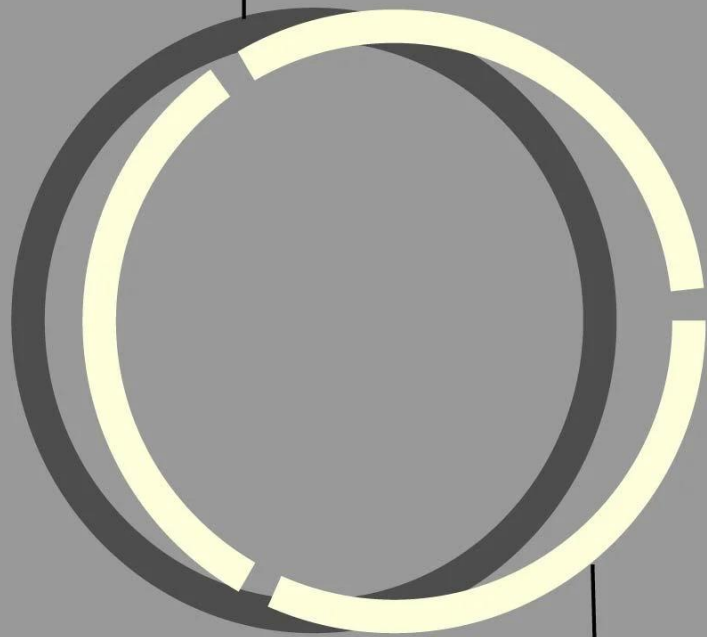
- specimen introduces a retardation of  $\lambda/4$
- phase plate introduces a retardation of  $\lambda/4$  for diffracted rays
- together there is a retardation of  $\lambda/2$
- destructive interference will change amplitude of wave and allow specimen to be seen



Left half only shown here ...

# Align Condenser Annulus

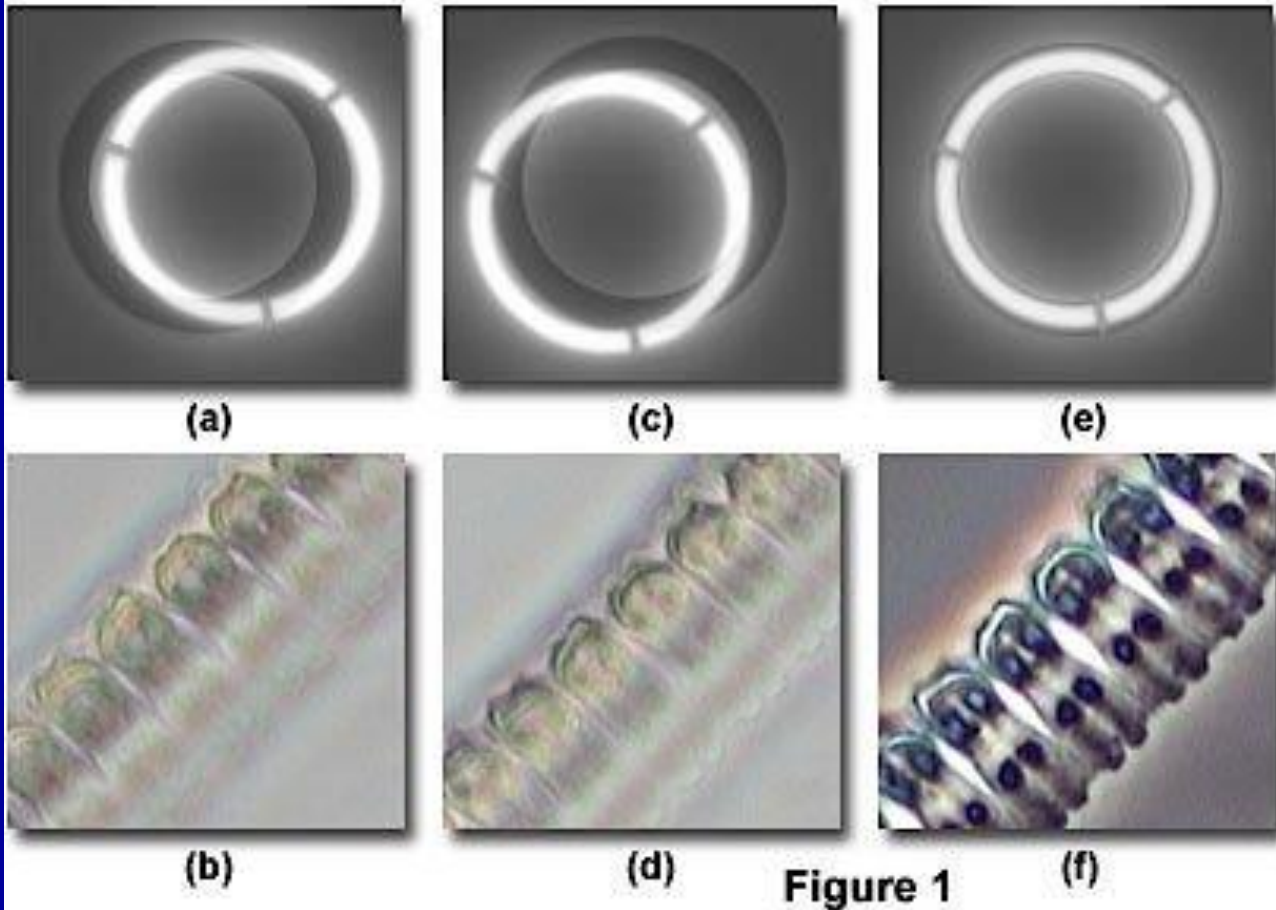
Objective phase plate



Condenser annulus

# Fully Aligned !

Phase Contrast Optical System Alignment

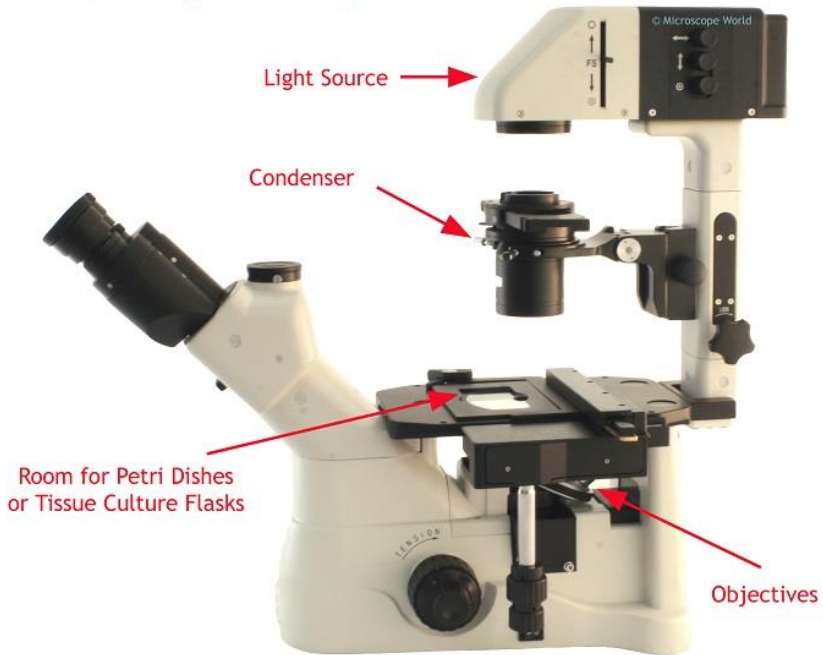


Phase Telescope  
or  
Bertrand Lens

# Inverted Microscope

View live cells in petri dish etc ...

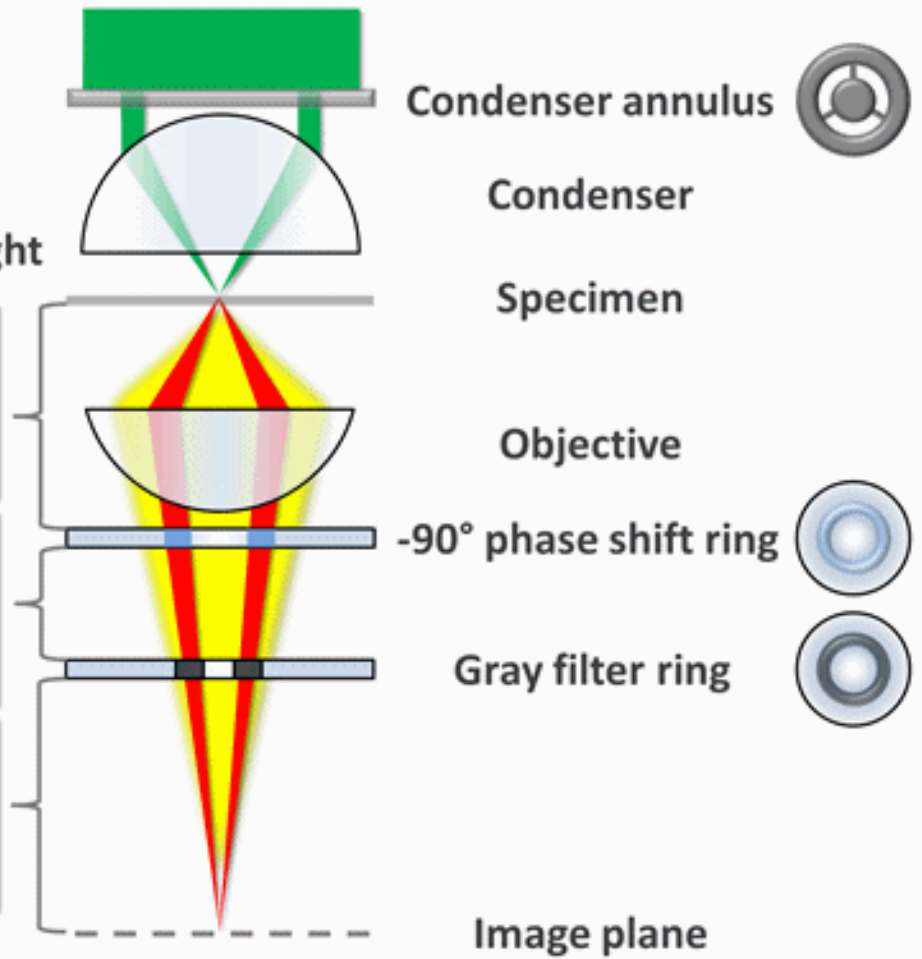
Inverted Biological Microscope



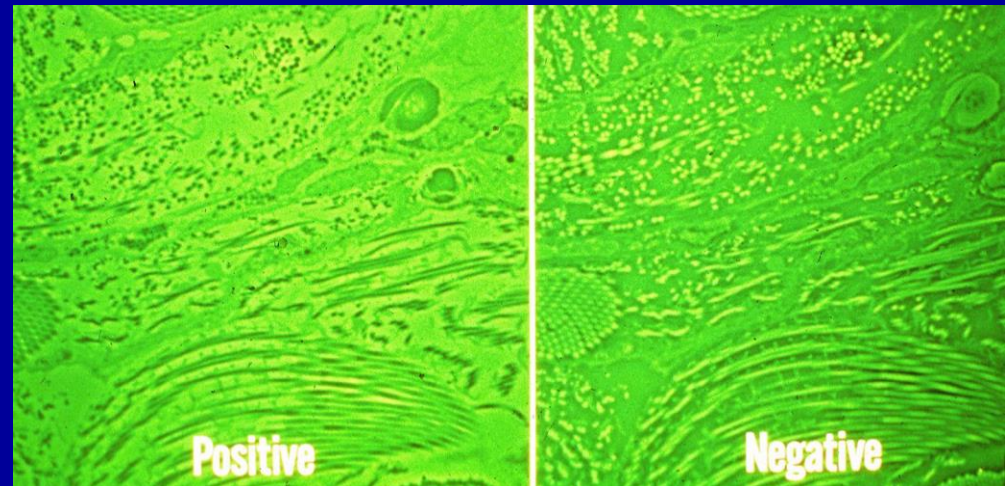
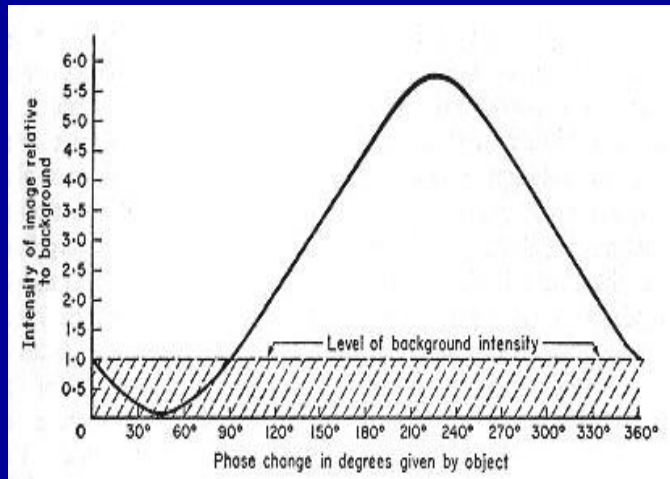
- Illuminating light
- Background light
- Specimen scattered light
- Foreground = background + scattered light

- Scattered light  $-90^\circ$  phase shifted
- (a) foreground  $\approx$  background
- Background light  $-90^\circ$  phase shifted
- (b) foreground  $>$  background
- Background light dimmed
- (c) foreground  $\gg$  background

Vector length and direction respectively corresponds to light intensity and phase difference



# Phase contrast microscope 5



- small phase changes – objects darker than background
- larger phase changes – objects brighter than background
- possibility of negative phase contrast microscopes



# Phase Contrast (Positive / Negative)

Figure 10.8 Cross sections of different types of phase plates. Gray = longer path length, Green = light absorbing material.

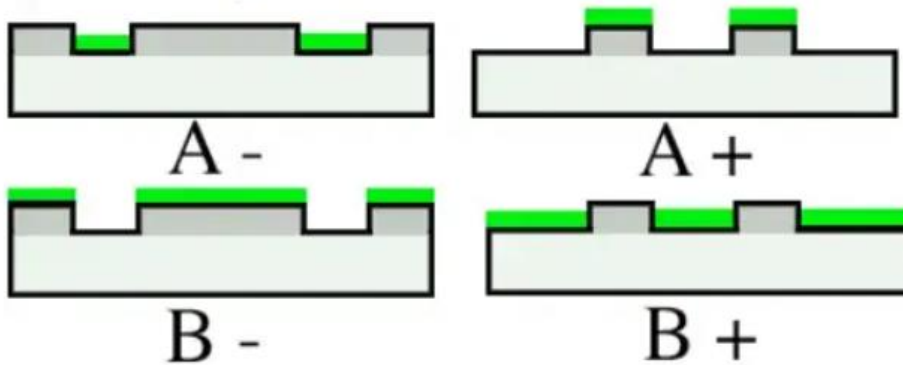
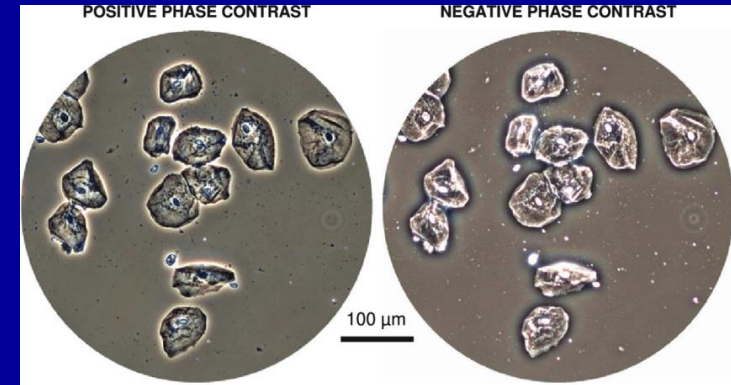


Fig: Cross sections of different types of phase plates. Gray = longer path length, Green = light absorbing material.



## Positive PC

## Negative PC

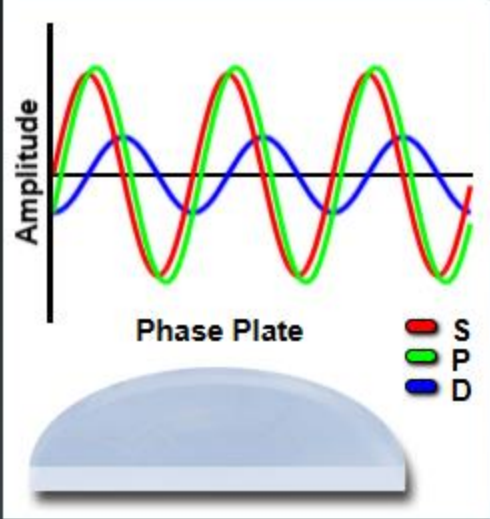
1. In **"positive" phase contrast**, the phase of the direct light is advanced by 1/4 wave (- type). This results in destructive interference and dark details on a bright background. The most prevalent form of phase contrast.
2. In **"negative" phase contrast**, direct light is slowed by a quarter-wave in phase (+ type). This results in luminous details on a dark background due to constructive interference.
3. In **positive or negative phase contrast**, the phase plate might be one of two varieties. Either the straight light (type A) or the diffracted light (type B) can be absorbed (B type). A - is the most prevalent type. Both A and B type plates are assessed by the transmission percentage of the ring area, with 20 percent being the norm.

+Coatings -75%  
+Apodized ND filters

[Phase Contrast Microscopy: definition, parts, uses, working principle. - Biology Notes Online](#)

# Online Interactive Tutorials

## Positive and Negative Phase Contrast

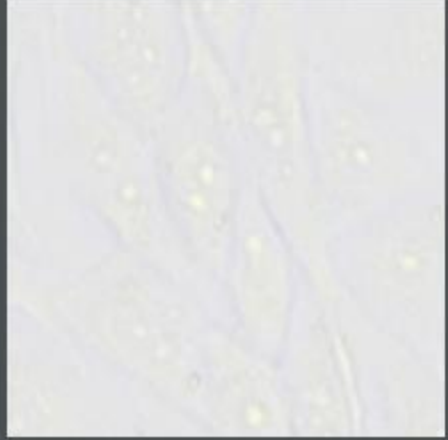


The graph shows three sinusoidal waves: a red wave (S), a green wave (P), and a blue wave (D). The y-axis is labeled 'Amplitude'. The red and green waves are in phase with each other but out of phase with the blue wave. Below the graph is a diagram of a phase plate, which is a semi-circular lens-like structure.

Phase Plate

- S
- P
- D

### Phase Contrast Image



A grayscale phase contrast image showing several bright, circular spots (nuclei) against a lighter background, representing tissue culture cells.

### Phase Contrast Mode

Negative Phase Contrast

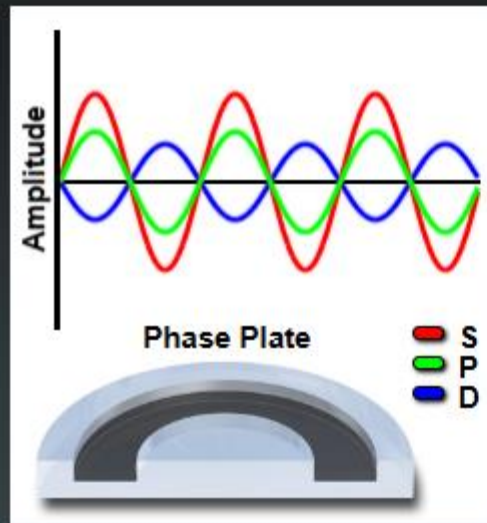
**Brightfield**

Positive Phase Contrast

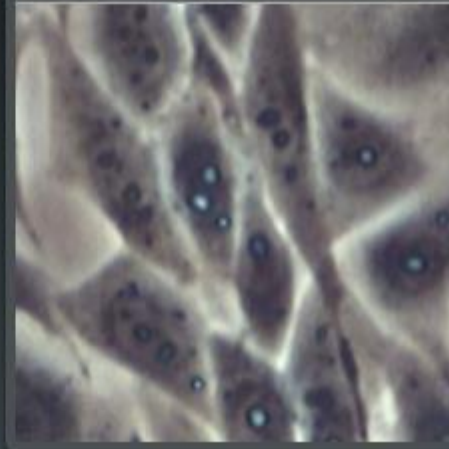
### Choose a Specimen

Tissue Culture Cells ▾

## Positive and Negative Phase Contrast



Phase Contrast Image



### Phase Contrast Mode

Negative Phase Contrast

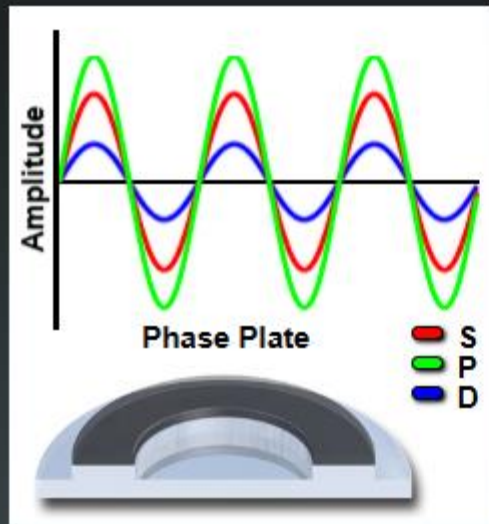
Brightfield

Positive Phase Contrast

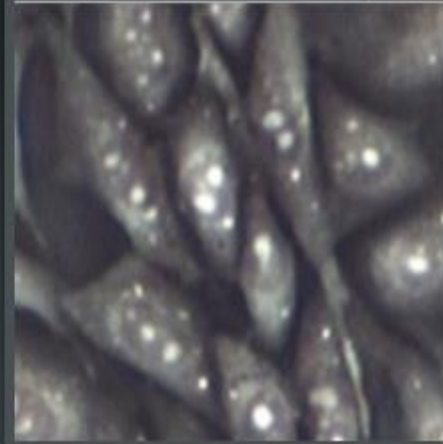
### Choose a Specimen

Tissue Culture Cells ▾

## Positive and Negative Phase Contrast



Phase Contrast Image



### Phase Contrast Mode

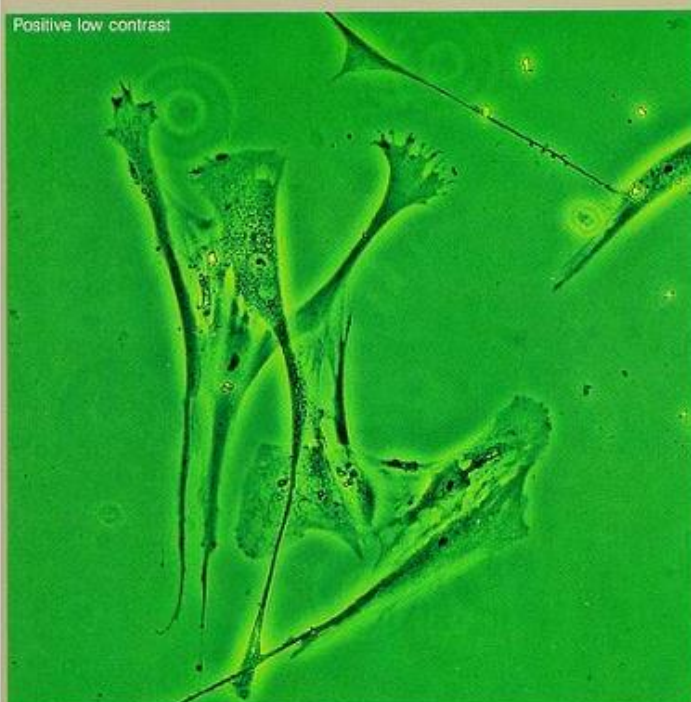
Negative Phase Contrast

Brightfield

Positive Phase Contrast

### Choose a Specimen

Tissue Culture Cells ▾



▲Fibroblast (Human embryo). PC D 20XPL, NFK 2.5XLD.



▲PC D 20XPLL, NFK 2.5XLD.



▲PC D 20XNML, NFK 2.5XLD.



▲PC D 20XNM, NFK 2.5XLD.

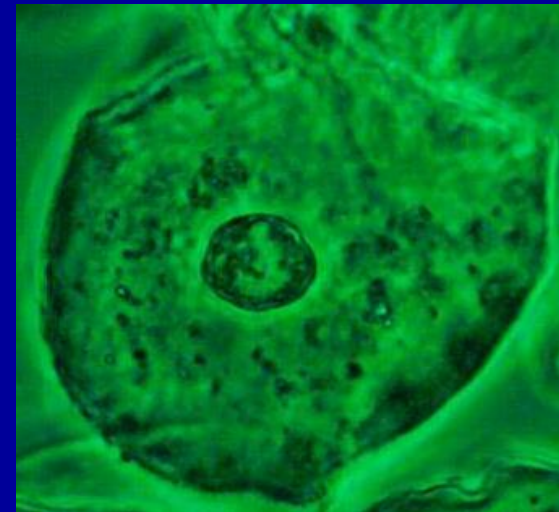
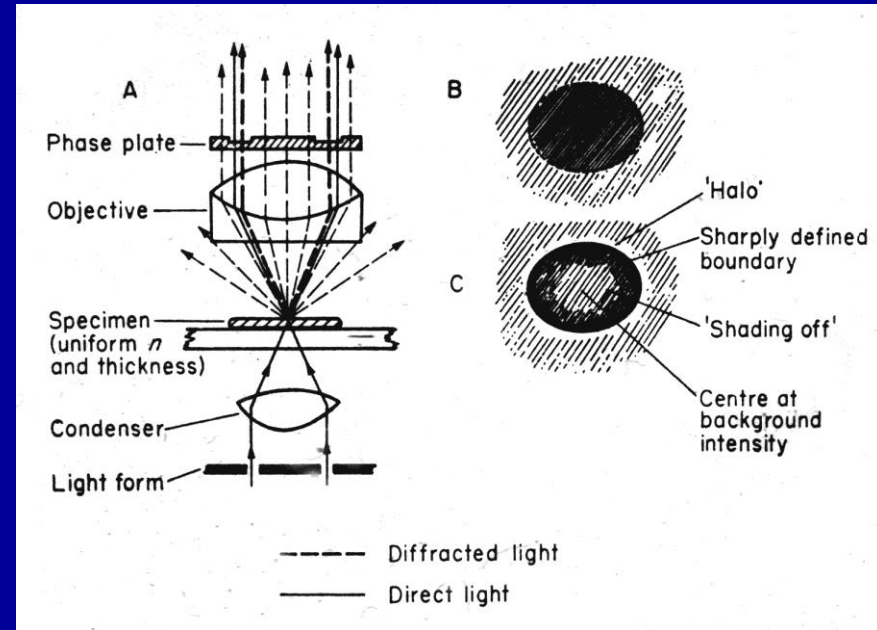


# Phase contrast microscope 6

## Problems/Artefacts:

due to incomplete separation of rays

- halos
- shading off
- contrast inversion (OPD)
- colours
- Stained = 'muddy'
- Use Thin, Unstained, + Green (550nm) filter to increase contrast



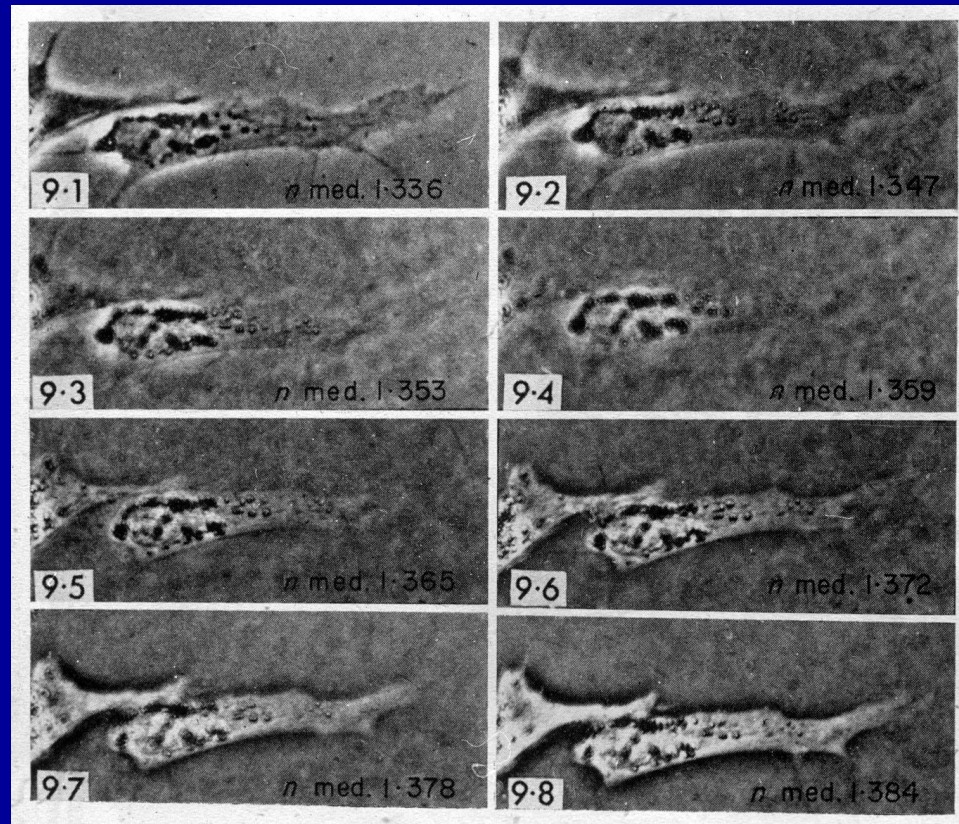
# Quantitative Microscopy

Measure alterations in light ...	Specimen	Microscope	Quantitation
Direction	Opaque material	Reflectance SEM	Count silver grains Autoradiography
Amplitude Amplitude ( $\lambda$ )	Dyes > contrast Dyes > colour	TEM BF LM	Morphometry Stereology Eyescales, Photometry Microdensitometry Microspectrophotometry
Wavelength	Fluorescent X-ray emission	Fluorescence Electron probe microanalysis	Fluorometry Photometry
Plane of Vibration	Anisotropy Birefringence	Polarising	Macromol arrangement Refractive index
Phase change	Living cells R.I. & Diffraction	Phase contrast	Immersion refractometry
Phase change	R.I. & Thickness	Interference	Thickness, Mass, Weight

See Quantitation Lecture ...



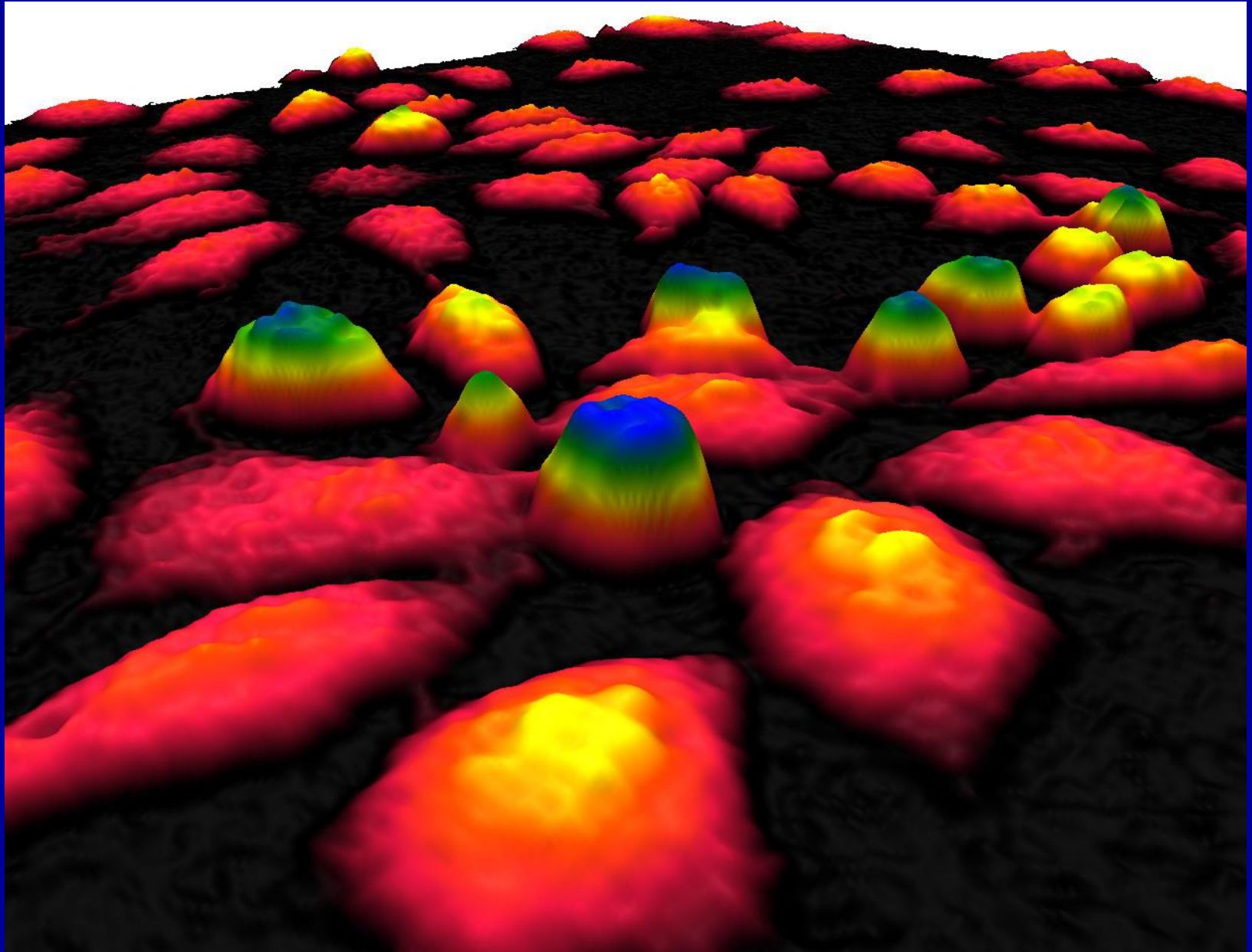
# Phase contrast microscope 4



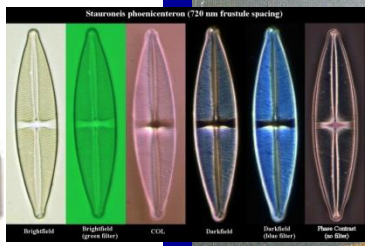
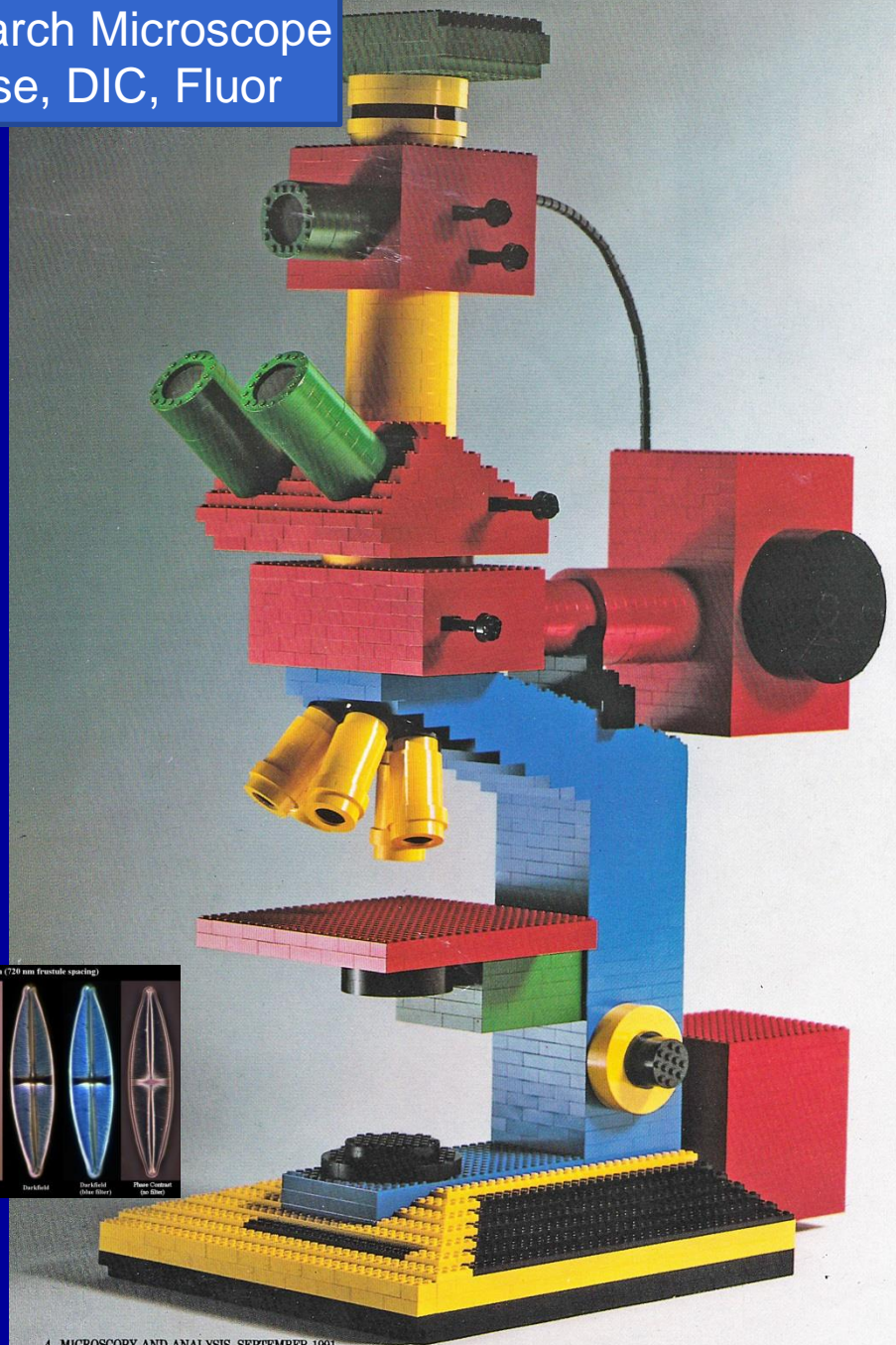
Immersion  
Refractometry

- refractive index of mounting medium is important
- changing it changes contrast
- **Barer (1950s)** first used it to quantify density, mass

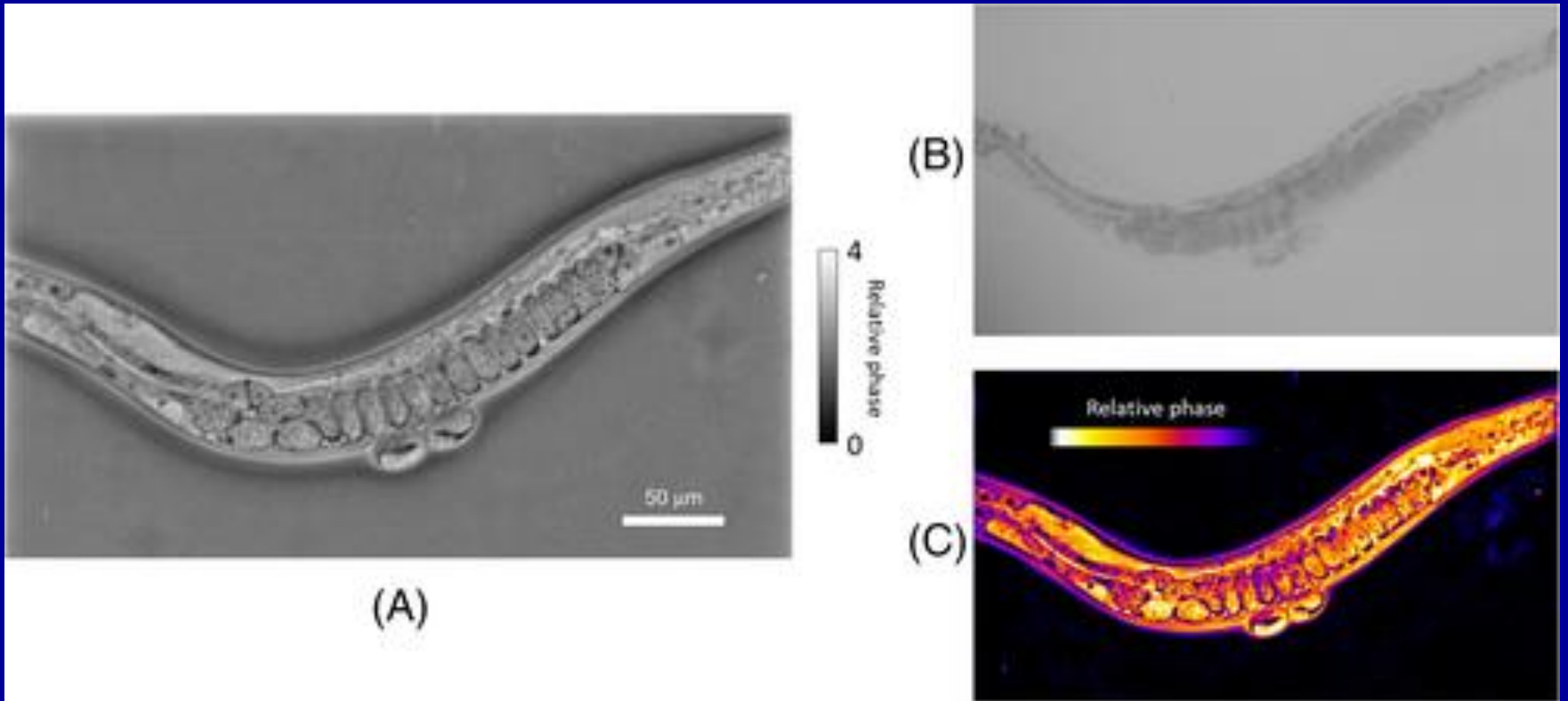
'Quantitative' Phase Contrast – Optical thickness is colour coded (Wikipedia)



# Modular Research Microscope BF, DF, Phase, DIC, Fluor



# Phase plus DIC



# Phase + Fluorescence

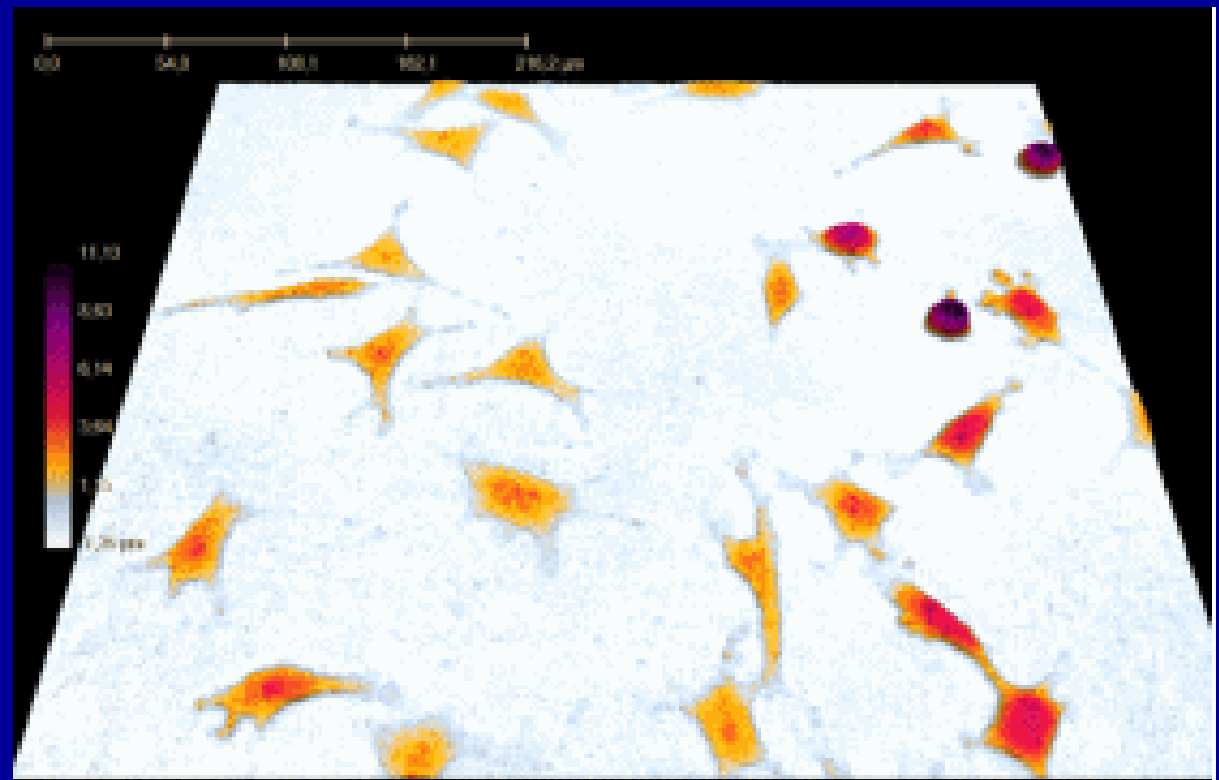
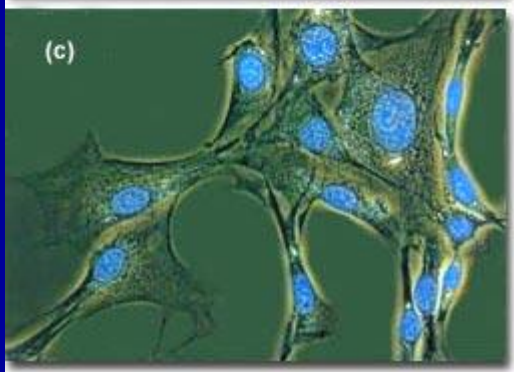
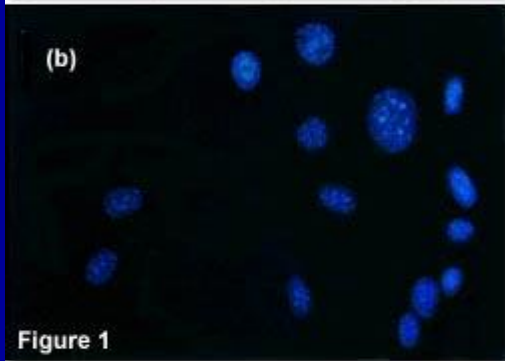
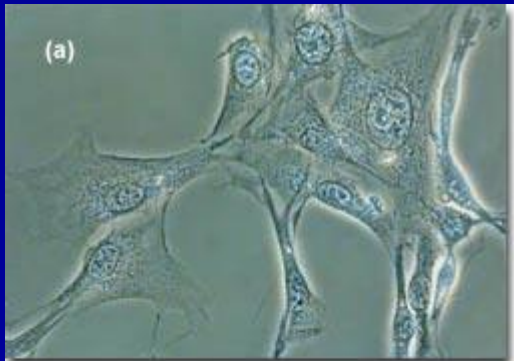
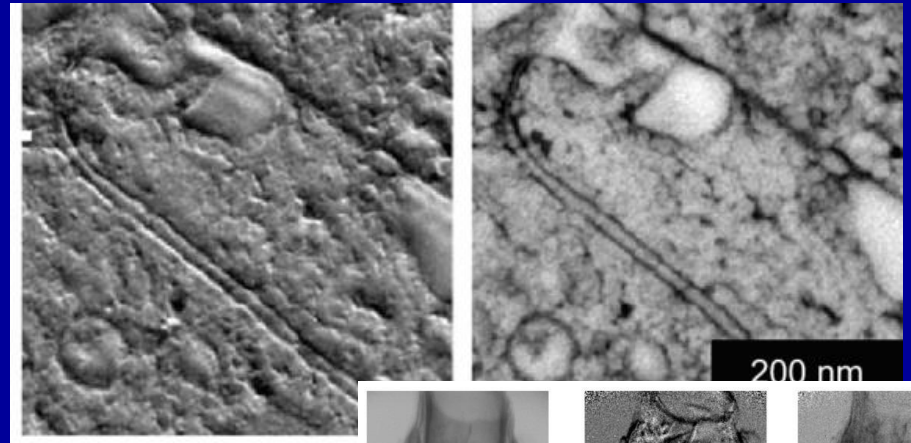


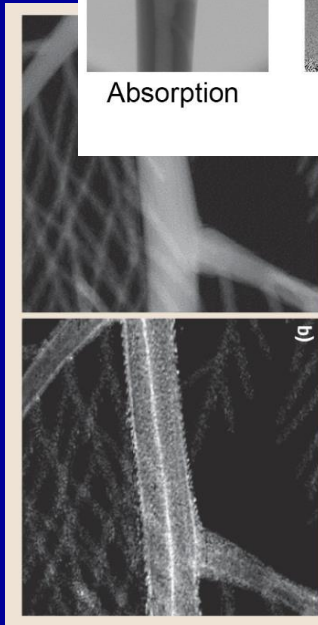
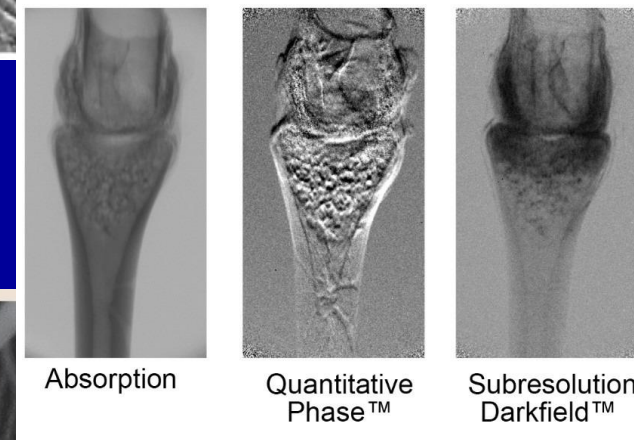
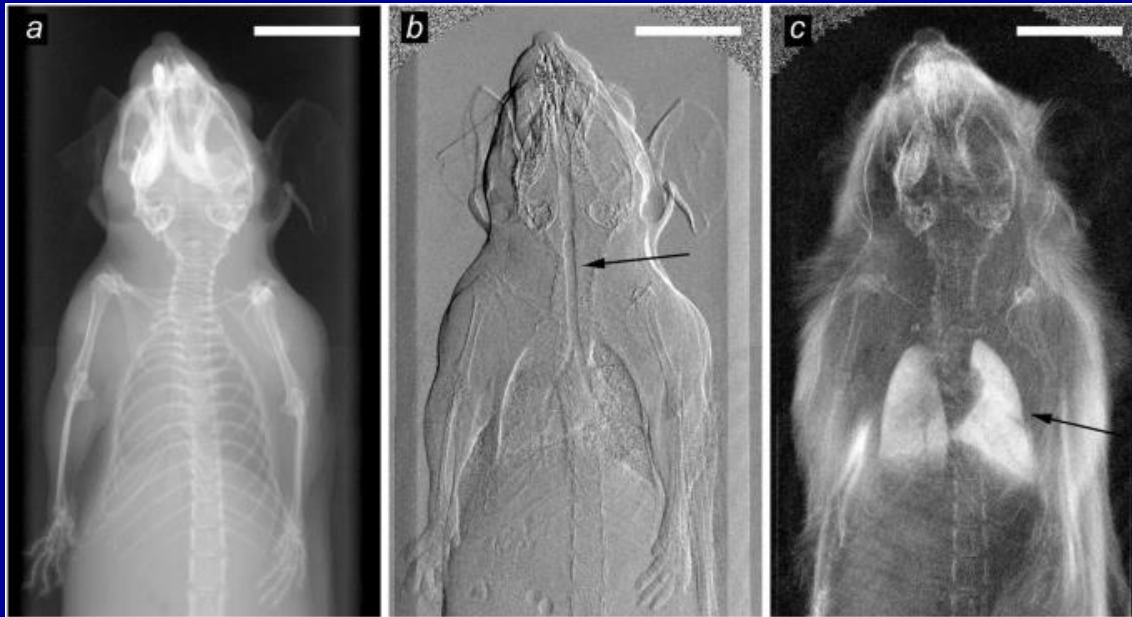
Figure 1

# Recent ...

Phase contrast electron microscopy



Phase X-ray microscopy



# SUMMARY: Contrast Enhancement

- Staining (dyes, metals, fluorophores), media
- Setup  $\Delta$  – out of focus (Becke), condenser position
- Oblique illumination
- Dark Field
- Rheinberg, Spikeberg, VAC
- Dispersion ‘Staining’
- Polarising
- **Phase Contrast, Leitz Heine PC Condenser**
- Hoffman Modulation Contrast, L IMC, NAHC, O RC, Z Varel
- Interference Contrast, DIC
- Colour Filters, Photoshop !!

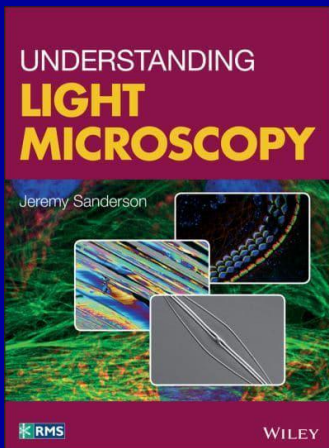
# References

[Phase-contrast microscopy - Wikipedia](#)

[BW OPTICS](#)

[Introduction to Phase Contrast Microscopy | Nikon's MicroscopyU](#)

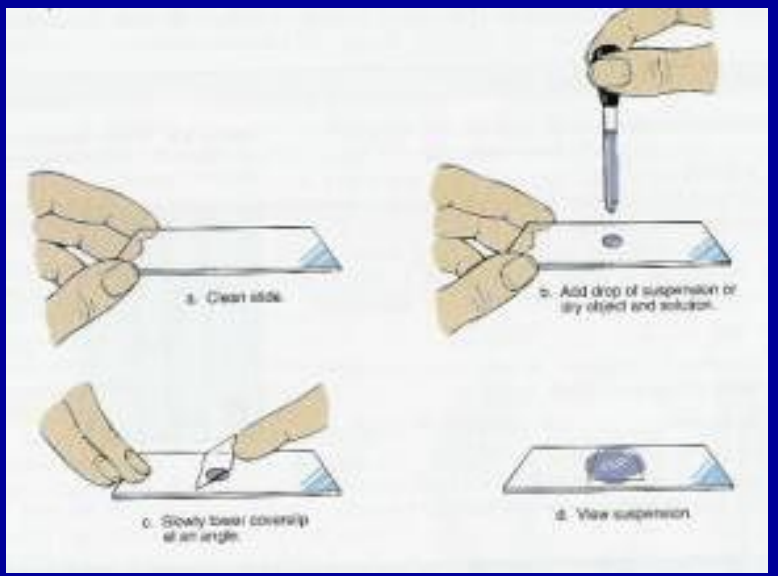
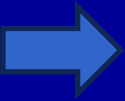
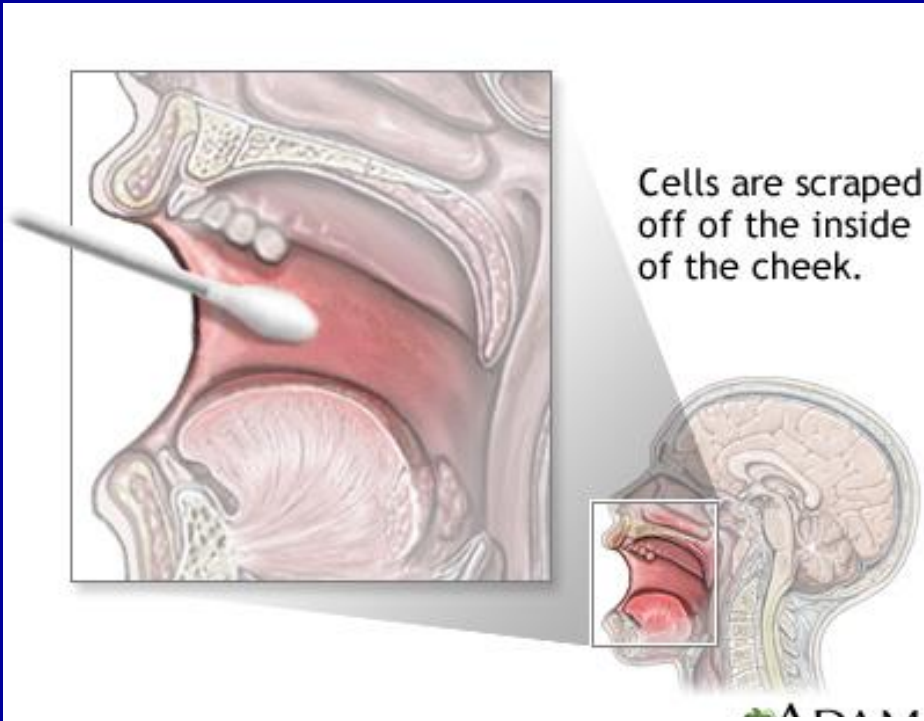
[Phase Contrast Microscopy - Introduction | Olympus LS](#)



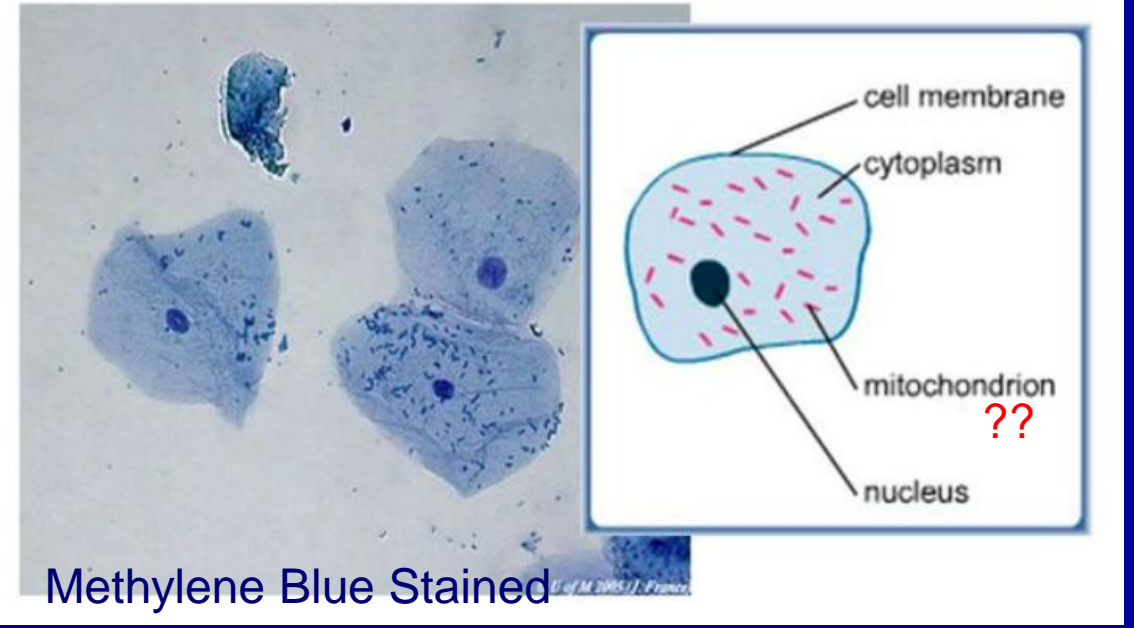


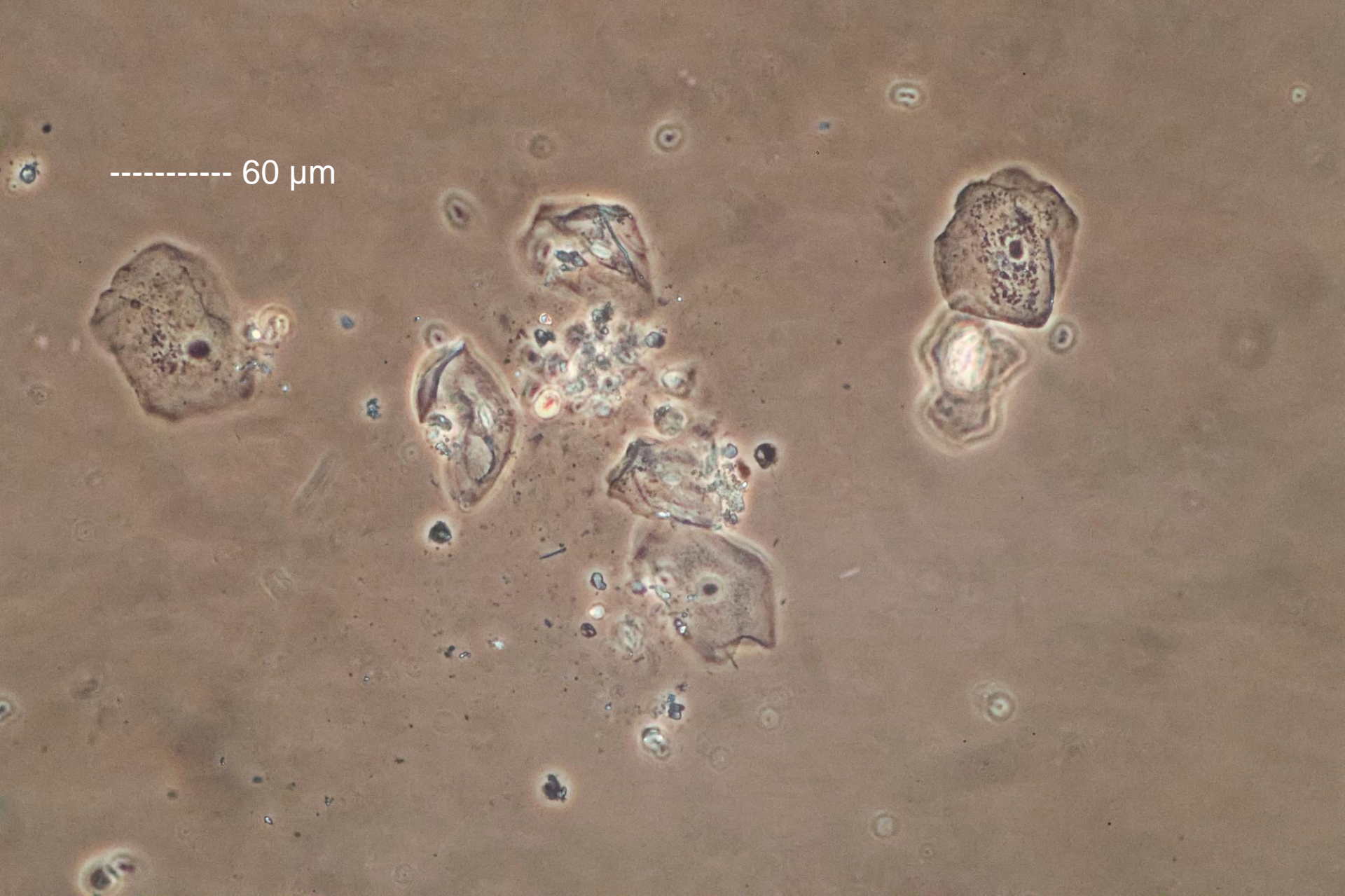
# Practical

- **View Phase Contrast microscopes**
  - Olympus
  - Lomo
- **Prepared slides**
  - Cheek cells
  - Pond water organisms
  - Diatoms
  - ...
- **Prepare own cheek smears & view with Phase Contrast**
- **At home**
  - view links to videos & references
  - Prepare cheek smears – stain with methylene blue



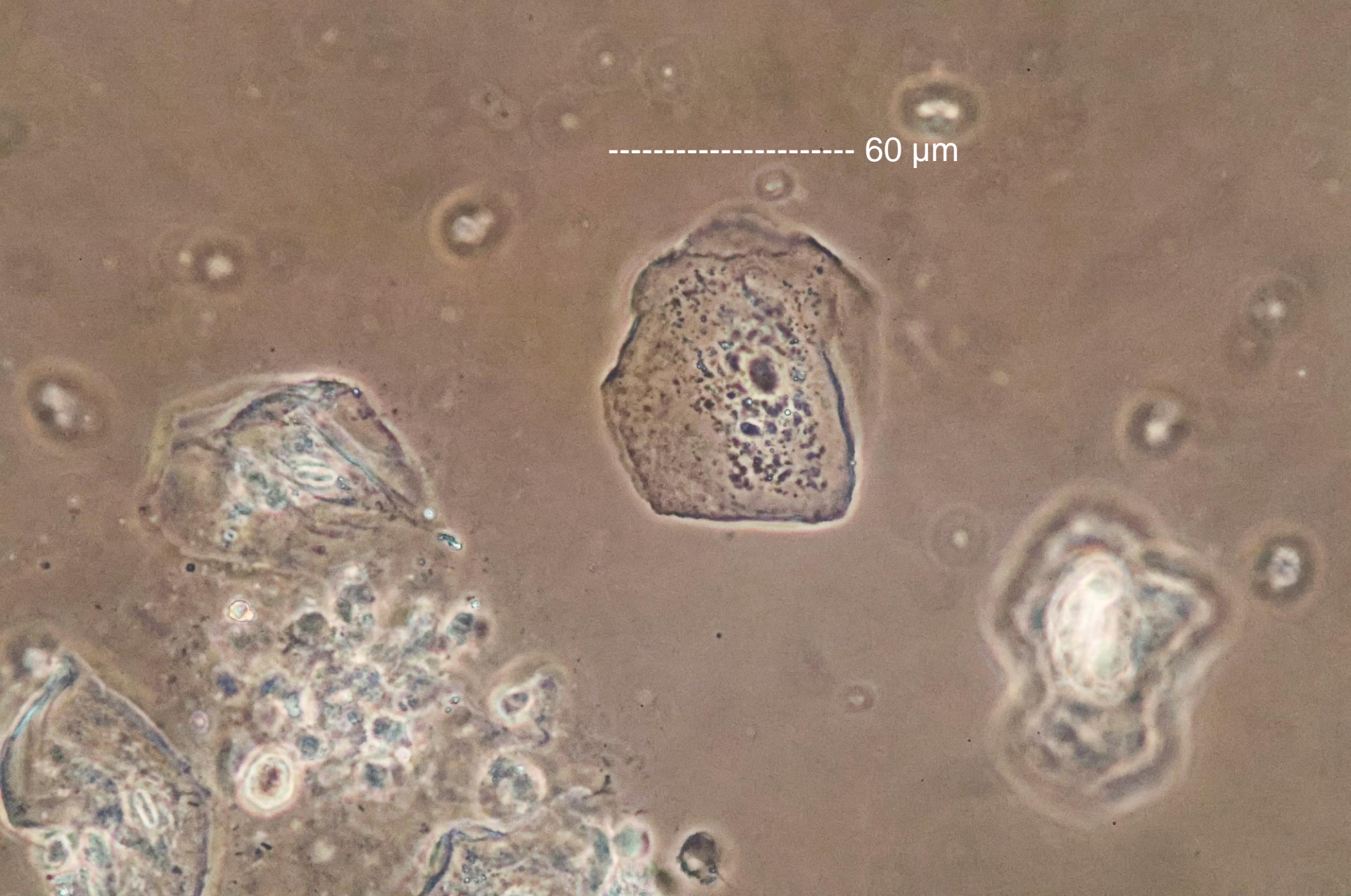
Try and get as close to this appearance **without** fixing/staining





----- 60  $\mu\text{m}$

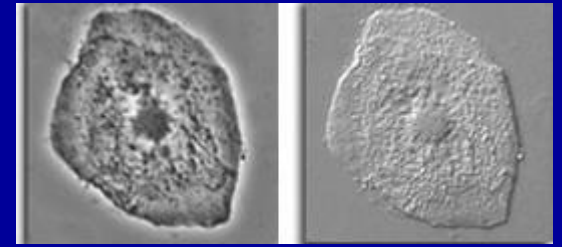
MM cheek cells – Phase Contrast x20



----- 60  $\mu$ m

MM cheek cells – Phase Contrast x40

# Types of Microscopy



Bright field

Dark Field/Refl

Polarising

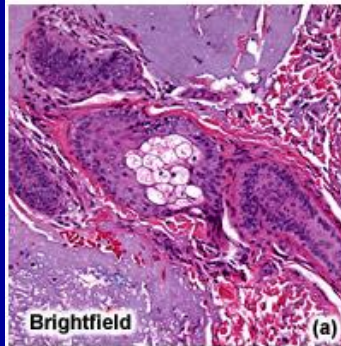
**Phase Contrast**

Interference

Fluorescence

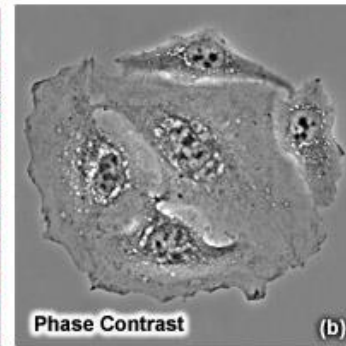


Contrast-Enhancing Techniques in Optical Microscopy



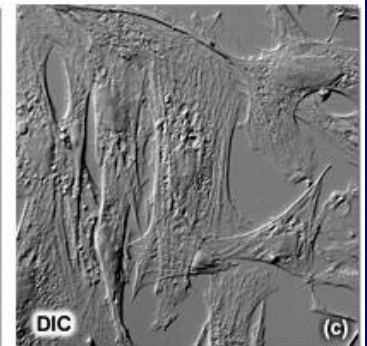
Brightfield

(a)



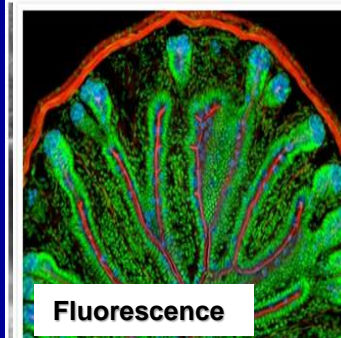
Phase Contrast

(b)

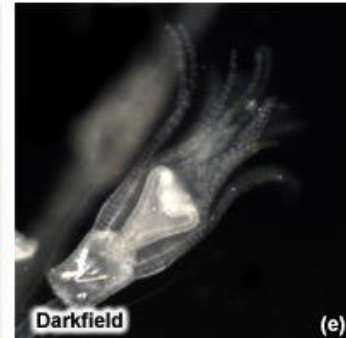


DIC

(c)

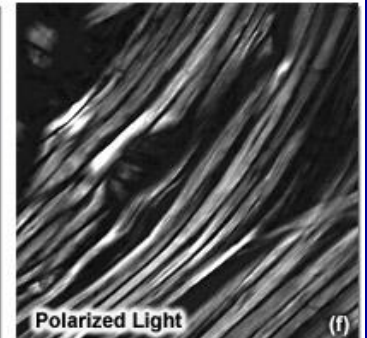


Fluorescence



Darkfield

(e)



Polarized Light

(f)

# Microscopy on Postage Stamps

